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Maria Soares Cachide de Almeida

Estudos moleculares do *BIN1* num coorte baseado em cuidados primários

BIN1 molecular studies in a primary care-based cohort

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Tese apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biomedicina Molecular, realizada sob a orientação científica da Professora Doutora Odete Abreu Beirão da Cruz e Silva, Professora Auxiliar com Agregação do Departamento de Ciências Médicas da Universidade de Aveiro.

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o júri

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palavras-chave

Demência; Doença de Alzheimer ; *BIN1*; Genética Molecular; Polimorfismo; Envelhecimento; População Portuguesa

resumo

Demência é uma síndrome clínica caracterizada pelo declínio progressivo das capacidades cognitivas, estando a tornar-se cada vez mais comum, devido ao envelhecimento da população mundial. Prevê-se que o número de doentes com demência aumente em cerca de 30 milhões nos próximos 15 anos, representando grandes gastos para os sistemas de saúde e sociais. Existem vários tipos de demência, sendo que a Doença de Alzheimer (DA) é a mais comum, afetando entre 20 a 30 milhões de pessoas em todo o mundo, das quais 90.000 são portuguesas. A compreensão das características genéticas e moleculares associadas a esta doença pode constituir um meio para descobrir novos métodos de diagnóstico e tratamento. A maior parte dos casos de Alzheimer tem início tardio, afetando indivíduos com 65 ou mais anos de idade. Até recentemente apenas o gene que codifica a Apolipoproteína E (*APOE*) foi associado com esta forma de DA. No entanto, estudos recentes de associação genómica identificaram o gene *BIN1* como sendo o loci de risco associado ao Alzheimer tardio mais significativo depois do *APOE*. Além disso, vários SNPs do *BIN1* foram associados a este tipo de Alzheimer, sendo que o polimorfismo rs744373 foi proposto como um dos mais relevantes para a DA. Dado que os SNPs mais significativos podem variar de população para população, o objetivo principal deste trabalho foi avaliar se o polimorfismo rs744373 do gene *BIN1* pode ser associado a um aumento do risco de desenvolver DA, numa população portuguesa do distrito de Aveiro, que pertence a um estudo transversal baseado em populações, realizado na Universidade de Aveiro. Analisámos 63 indivíduos Portugueses, sendo 32 doentes e 31 controlos. Neste estudo conseguimos observar que, de uma forma geral, o alelo A é o mais frequente e que o alelo G (alelo de risco) foi o menos frequente, numa razão de 3:1. Não conseguimos encontrar uma forte evidência de associação entre o rs744373 e o risco de desenvolver DA (Razão de probabilidade [RP] = 0.733 , valor p = 0.464), o que está de acordo com estudos previamente publicados. Não foi detetada significância estatística entre o rs744373 e portadores do alelo APOE-Ɛ4 (valor p = 0.467) ou indivíduos com demência (CDR≥1) (valor p = 0.269). Foi detetada uma associação entre o alelo de risco do polimorfismo de estudo e a presença de Diabetes Mellitus (RP = 6.60, valor-p = 0.035). No entanto, como a nossa amostra era pequena, deve ser feito um novo estudo para avaliar se este resultado pode ser generalizado para a população Portuguesa.

keywords

Dementia; Alzheimer's Disease; *BIN1*; Molecular Genetics; Polymorphism; Aging; Portuguese population

abstract

Dementia is a clinical syndrome characterized by a progressive decline in cognitive skills, and is becoming increasingly common, due to the aging of the world's population. It is expected that the number of patients with dementia will increase by 30 million in the next 15 years, representing a major factor of costs in health care and social systems. There are several types of dementia, and Alzheimer's Disease (AD) is the most common, affecting 20 to 30 million people worldwide, of which 90.000 are Portuguese. Understanding the genetic and molecular characteristics associated with the disease may constitute a way to discover new diagnostic methods and treatments. Most cases of AD are late-onset (LOAD), affecting individuals with 65 or more years of age. Until recently only the Apolipoprotein E gene (*APOE*) had been associated with this form of AD. However, recent genome-wide association studies have identified Bridging Integrator 1 (*BIN1*) as the most significant LOAD-associated risk loci after *APOE*. Furthermore, several SNPs of *BIN1* have been associated to this type of AD and rs744373 was proposed to be one of the most relevant for AD. Since the most significant SNP may vary from population to population, the main aim of this work was to evaluate if *BIN1* polymorphism rs744373 can be associated with the risk of AD in a Portuguese population from the Aveiro district, belonging to a cross-sectional population-based study performed in Aveiro University. We analysed 63 Portuguese individuals comprising 32 cases and 31 controls. In this study we could observe that, overall, allele A was the most frequent and allele G (risk allele) was the least frequent, in a ratio of 3:1. We didn't find strong evidence of association for rs744373 with the AD risk (odds ratio [OR] = 0.733 , p-value = 0.464), which is in agreement with some previous published studies. No statistical significance was detected between rs744373 and APOE-Ɛ4 carriers (p-value = 0.467) or individuals with dementia (CDR≥1) (p value= 0.269). We have detected an association between the risk allele of the study polymorphism and the presence of Diabetes Mellitus (odds ratio [OR] = 6.60, p-p-value = 0.035). Nevertheless, due to our small sample size, a follow-up study is required in order to evaluate if this result can be generalized to the Portuguese population.

INDEX

ABBREVIATIONS

AD	Alzheimer´s Disease
AGD	Angyrophilic Grain Disease
AICD	Amyloid Precursor Protein Intracellular Domain
APP	Amyloid Precursor Protein
APOE	Apolipoprotein E
Aβ	Amyloid-β peptides
BAR	*BIN1*/amphiphysin/RVS167
BIN1	Bridging Integrator 1
CBD	Corticobasal Degeneration
CDK5	Cyclin Dependent Kinase 5
CDR	Clinical Dementia Rate
c-MYC	c-myelocytomatosis
CNS	Central Nervous System
CSF	Cerebrospinal Fluid
DM	Diabetes Mellitus
EC	Extracellular Domain
EOAD	Early-Onset Alzheimer's Disease
GSK-3β	Glycogen Synthase Kinase 3β
GWAS	Genome-Wide Association Studies
IC	APP Intracellular Domain
MBD	Myc-Binding Domain
MCI	Mild Cognitive Impairment
MiBD	Microtubule-Binding Domain
MRI	Magnetic Resonance Imaging
NFT	Neurofibrillary Tangle
PD	Projection Domain
PET	Positron Emission Tomography

PHF	Paired Helical Filament
PiB	Pittsburgh Compound B
PiD	Pick's Disease
PP2A	Protein Phosphatase 2A
PSEN1	Presenilin 1
PSEN2	Presenilin 2
PSP	Progressive Supranuclear Palsy
SH3	Src Homology 3
SNP	Single Nucleotide Polymorphism
SP	Senile Plaque
TM	Transmembrane Domain

1. INTRODUCTION

1.1 Dementia

Dementia is a clinical syndrome usually associated with a group of symptoms and signs that culminates with a progressive decline in cognitive skills such as memory, reasoning, attention and orientation. It is also characterized by difficulty in language processing, as well as emotional and social changes, which might be linked to a state of depression, agitation, hallucinations, disinhibition or even insomnia. These changes impair daily life activities [1] like driving, shopping, cooking or even attending to personal care, which makes it very hard for patient's caregivers [2,3]. With the world's population rapidly aging, it's expected that the number of patients with dementia will increase 30 million in 15 years [3].

Depending on the cause, dementia can be reversible (rare and potentially treatable) or irreversible, being the first a result of other medical condition and the latter a consequence of a neurodegenerative process [3]. Either way, most forms of dementia are progressive, becoming more severe as the patient ages. However, it is important to highlight that dementia is not part of the natural aging process, and so not all old people will develop the symptoms associated to it. Actually, although dementia is most frequent after the age of 65, there are cases of patients in the age group of 40 to 60 years old that suffer from it [4]. For these reasons, it is still unclear if dementia is only an aging-related condition or if it tends to develop in older people just because it requires time for the pathogenic process to disclose [5].

There are several types of dementia including Parkinson's disease [6], dementia with Lewis bodies, Frontotemporal dementia and Alzheimer's Disease (AD) [3]. Depending on the dementia, although they all tend to have a protracted development, there are two patterns of decline, being the first characterized by the late onset of clinical symptoms, while the other presents at an early stage.

Since Alzheimer's disease is the most common form of age-related dementia, the next sections of the present work will be an overview of its molecular and genetic basis.

1.2 Alzheimer's Disease (AD)

AD is an irreversible and progressive brain disorder, where neurodegeneration is its main feature [7,8]. It is characterized by the impairment of several cognitive functions such as memory, attention, concentration, language as well as emotional and social changes. In the final stages of the disease such symptoms are so severe that the patient has no autonomy, requiring a full-time caregiver. In these patients, pneumonia tends to be the leading cause of death [9].

It is one of the most common diseases in the developed world [10], having an incidence of about 20 to 30 million people worldwide [11–13], with an estimated increase of more than 50 million people by the year 2040 [14]. Currently, in Portugal nearly 90.000 individuals suffer from AD leading to consider it a major driver of costs in health care and social systems [4].

The first case of AD was described in 1906 by the German psychiatrist and neuropathologist Dr. Alois Alzheimer after whom the disease was named, and it referred to an unusual disease of the cerebral cortex of a 51-year-old woman (Auguste D.). This case was an early onset, potentially caused by a PSEN2 Volga-German mutation, which led to a progressive cognitive impairment with loss of memory and language skills as well as behavioural changes [3,14].

AD is a multifactorial dementia caused by genetic, epigenetic and environmental factors and pathways, which interact among themselves leading to a complex heterogeneity of patient populations [5,15]. Some of the non-genetic factors that might contribute to the development of the disease include infections, hormones, diabetes, smoking or even emotional and social factors [16].

Although the ultimate risk factor for the appearance of AD is age [3], it has been hypothesized that the development of this highly prevalent dementia begins 20 to 30 years before the manifestation of the first symptoms [11,17]. This first stage of the disease is called Mild Cognitive Impairment (MCI) in which people show some cognition deficits in comparison to the average individuals of their age, although it does not interfere with their daily lives in a significant way. The transition to AD occurs when the neuropathological features are more relevant, such as the spread of neurofibrillary tangles (NFTs) beyond the medial temporal lobe in cortex [7,11].

1.3 Neuropathological Hallmarks of AD

Neuropathologically, AD is characterized by atypical protein aggregates [18]. The main hallmarks of the disease include the presence of senile plaques (SP) and neurofibrillary tangles (NFTs), being the first an extracellular accumulation of Amyloid-β peptides (Aβ) and the latter a intraneuronal accumulation of hyperphosphorylated TAU proteins [19,20] (Figure 1). Neurofilaments (NFs) may also play an important role in neurofibrillary pathology and degeneration as they are one of the key components of the neuronal cytoskeleton, being the most abundant cytoskeletal protein in large myelinated axons [21].

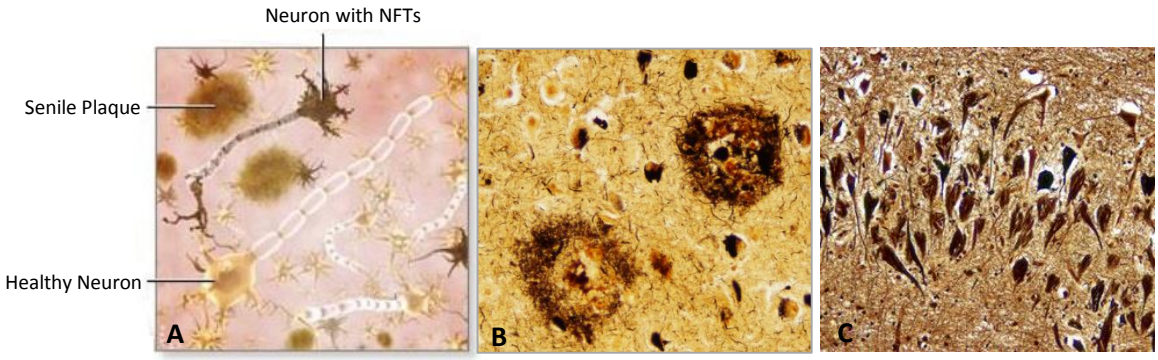

Figure 1 – Neuropathological Hallmarks of AD. A - Schematic representation of the neuropathological hallmarks of AD. **B -** Senile Plaques (SP) (seen with Bielschowski silver stain). **C –** Neurofibrillary tangles (NFTs) (seen with Bielschowski silver stain). (Taken from[22,23])

Another feature of AD is synapse loss, which may be related to changes in the normal functioning of neurons or even due to neuron death. This all culminates in brain atrophy [24,25] of specific regions involved in memory and learning processes, namely the limbic system [26,27], neocortical regions [28], the basal forebrain [29], and the hippocampus [30], which may partly explain the clinical symptoms characteristic of this disease (Figure 2).

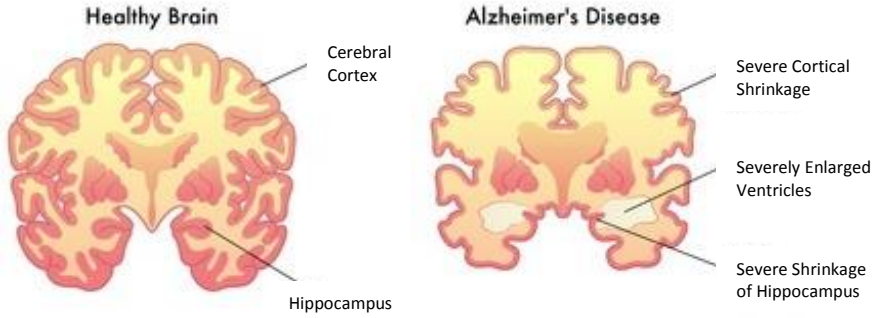

Figure 2 – Schematic comparison between a cross section of a healthy brain (left) and a brain of a patient with AD (right). In the latter it is possible to notice an overall shrinkage, mainly in the cortex and hippocampus, and an enlargement of the ventricles. (Taken from [31])

1.3.1 Senile Plaques (SP)

Senile Plaques are spherical lesions resulting from the build-up of extracellular Aβ, either with 40 amino acids ($A\beta_{40}$) or 42 amino acids ($A\beta_{42}$), which are produced through the metabolism of Amyloid Precursor Protein (APP) after being sequential cleaved by a group of specific secretases (β- and γ-secretases) [3,32]. There is a greater amount of $A\beta_{42}$ than $A\beta_{40}$ within the SP due to its higher rate of insolubility [33]. This neuropathological feature of AD, unlike NFTs, is found mainly in the isocortex [33].

1.3.1.1 Amyloid Precursor Protein (APP)

APP is a ubiquitously expressed type 1 transmembrane glycoprotein composed by 695 to 770 amino acids, encoded by a single gene located on chromosome 21 (21q21.23) with a length of about 240kb and no less than 18 exons [34]. Through alternative splicing of exons 7, 8 and 15 of the APP mRNA several isoforms are synthesized, predominantly APP_{695}, APP_{751} and APP_{770}. The 695 isoform is the only one that lacks the Kunitz Protease Inhibitor (KPI) domain.

Regarding the structure, APP has three main domains, the Extracellular Domain (EC) which includes amino acid residues 1-699 in exons 1-17, the Transmembrane Domain (TM) which is composed by residues 700-723 in exons 17 and 18, and the APP Intracellular Domain (AICD) which comprises residues 724-770 in exons 17 and 18. The Aβ sequence, which originates the Aβ peptide of particular interest in AD, is located in exons 16 and 17 (amino acid residues 672-713), being part of both EC and TM [16].

APP can undergo two different proteolytic pathways: a non-amyloidogenic and an amyloidogenic [18]. In the non-amyloidogenic pathway, APP starts to suffer the action of α-secretase, resulting APPsα (a soluble APP derivative) and a α-carboxyl-terminal fragment (APP-CTFα). This latter fragment is subsequently cleaved by γ-secretase generating a non-toxic peptide (p3) and the Amyloid Precursor Protein Intracellular Domain (AICD). In this pathway, as α-secretase action takes place in the Aβ peptide sequence, it prevents the subsequent formation of Aβ peptide, whose aggregation causes SPs. On the other hand, in the amyloidogenic pathway, APP is first cleaved by β-secretase, releasing APPsβ and β-carboxyl-terminal fragment (APP-CTFβ). The APP-CTFβ is afterwards cleaved by γ-secretase producing Aβ peptide and the AICD [16,35] (Figure 3). BACE1 and BACE2 are the two secretases capable of cleaving APP at the β-site, however it is the first one that

acts as a key β-secretase in the brain, since it is the rate-limiting enzyme that starts the Aβ formation [36].

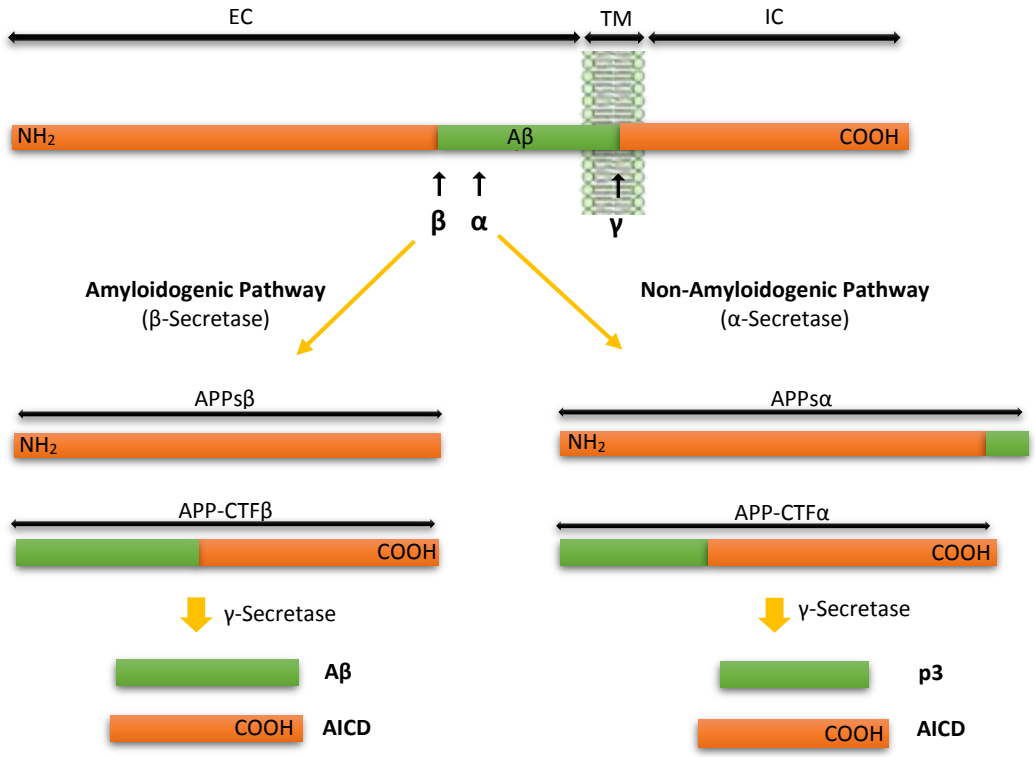

Figure 3 – Schematic diagram of APP processing pathways (not drawn to scale). In the **Amyloidogenic pathway** APP is cleaved by β-Secretase, originating APPsβ and APP-CTFβ. The latter is subsequently cleaved by γ-Secretase originating Aβ peptide and AICD fragment. On the other hand, in the **Non-Amyloidogenic pathway**, APP is first cleaved by α-Secretase, giving rise to APPsα and APP-CTFα, being the last cleaved by γ-Secretase, leading to the formation of p3 peptide and the AICD fragment. Abbreviations: EC, Extracellular Domain; TM, Transmembrane Domain; IC, Intracellular Domain. (Adapted from [16]).

1.3.1.2 Amyloid-β peptide (Aβ)

Amyloid β is a peptide with an approximate length of 40 to 42 amino acids [11], that is produced by proteolytic cleavage of APP, first identified in 1984 by Glenner and Wong [37].

Depending on the γ-secretase that cleaves APP, a variety of peptides can be generated, including $A\beta_{42}$ and $A\beta_{40}$. Of these, the $A\beta_{40}$ is the most abundant variant (about 80%)[35] present in both healthy brains and brains of people who suffer from AD. On the other hand, $A\beta_{42}$, which aggregates much easier when compared to $A\beta_{40}$, this is found to be augmented in neuronal cells which have suffered mutations in APP or Presenilin 1 (PSEN1) and Presenilin 2 (PSEN2) genes, which are associated with Early-Onset Alzheimer's Disease (EOAD) [38].

Aβ is normally secreted in physiological processes, which indicates that it has a physiological function [35]. Further, it has been discovered through several studies that Aβ is important in neurogenesis, producing neuroprotective effects and increasing cell viability when growth factors, neurotrophins or excitotoxic conditions are not present [39]. It also plays an important role in strengthening or weakening synapses, is relevant in synaptic plasticity [40] and memory formation [41]. In addition, it influences the metal sequestration and antioxidant activity with metals such as copper, zinc or iron. The Aβ peptide is also important for the maintenance of homeostasis and so it is particularly important in keeping the nervous system healthy [35]. The problem with Aβ occurs when there is an increase in the $A\beta_{42}/A\beta_{40}$ ratio, since it eventually contributes to the build-up of protein aggregates that accumulate in the brain, leading to neurodegeneration and the development of AD [16,35]. This is accordingly to amyloid hypothesis, which is well-known and accepted among the scientific community.

In addition, Aβ peptide accumulation might be toxic in a variety of ways, as it can induce oxidative stress when synchronized with redox active metals. It can also interact with membranes, leading to the formation of pores and consequently contributing to an abnormal flux of ions, culminating in neuron death. Finally, Aβ is associated with synaptic dysfunction, telomerase dysfunction (through its inhibition) or can even promote apoptosis of neuronal cells [35].

1.3.2 Neurofibrillary Tangles (NFTs)

NFTs consist of intraneuronal filamentous inclusions primarily constituted by aggregates of hyperphosphorylated TAU protein which, in a regular situation, has several roles including the stabilization of microtubules and intracellular transport, both axonal and vesicular. Normally TAU is a soluble protein, however, after suffering an excessive phosphorylation by several kinases eventually becomes insoluble, losing affinity to microtubules and self-associating into paired helical filament (PHF) structures and straight filaments, which ultimately evolve into NFTs [42]. The PHF breadth ranges from 8 to 20nm, with a spacing between crossovers of approximately 80 nm, while straight filaments do not have this helical periodicity [43]. The presence of NFTs can lead to impairment of the normal axonal transport of some components required for function and survival of neuronal cells, namely vesicles with neurotransmitters, mitochondria and neurotrophic factors, and may result in neurodegeneration [42].

NFTs are found in many other neurodegenerative diseases, of which the most predominant neuropathologic feature in Progressive Supranuclear Palsy (PSP), corticobasal degeneration (CBD), Pick's disease (PiD), Angyrophilic Grain Disease (AGD) and Dementia Pugilistica (DP), among others [44].

1.3.2.1 TAU protein

TAU is a microtubule-associated protein first discovered and isolated by Weingarten and colleagues in 1975 [45], having a major role in the assembly and stabilization of microtubules [46]. It is the major protein associated with the formation of NFTs.

- **Expression and isoforms of TAU**

Human TAU is encoded by the Microtubule-Associated Protein TAU gene (*MAPT*), which is located over 100kb on the long arm of chromosome 17, at position 17q21.1, comprising 16 exons [47]. Importantly, exon 1 belongs to the promoter and so, although transcribed it is not translated [48]. In normal adult human brain, alternative splicing of exon 2 (E2), exon 3 (E3) and exon 10 (E10) generates six TAU isoforms ranging from 352 to 441 amino acids in length. The size of those isoforms depends on the presence or absence of one or two 29- amino acid insert in the N-terminal, which are encoded by E2 and E3, and the inclusion or not of either three (R1, R3 and R4) or four (R1-R4) repeat regions in the C-terminal, which are encoded by E10. Therefore isoforms may be categorized according to the number of inserts encoded by E2 and E3 (0, 1 or 2), called 0N, 1N or 2N, and according to the number of repeat domains of the C-terminal encoded by E10 (3 or 4), called 3R or 4R [49] (Figure 4). The peripheral nervous system has neurons that often project a very long axon with a large diameter, due to the inclusion of a N-terminal sequence encoded by exon 4a, resulting in the expression of a specific TAU isoform named "big TAU" [50,51].

Throughout the development of the human brain, TAU isoforms are differentially expressed, whereby during the foetal stages only the shortest TAU isoform is present (0N3R), while during adulthood all six isoforms are found in the brain [50]. Also, foetal forms of TAU promote assembly of microtubules less actively than adult forms, since it is in the inter-region between R1 and R2 that relies the most potent part that induces microtubule polymerization. Notably this R1-R2 inter-region is characteristic of adult TAU isoforms 4R, being responsible for the difference in the binding affinities between 3R and 4R TAU [52].

Importantly, the alternative splicing of E10 is linked to different tauopathies according to the isoforms found in the protein aggregates, namely 4R tauopathies (comprising PSP, CBD and AGD), 3R tauopathies (such as PiD) and 3R+4R tauopathies, as exemplified by AD [53].

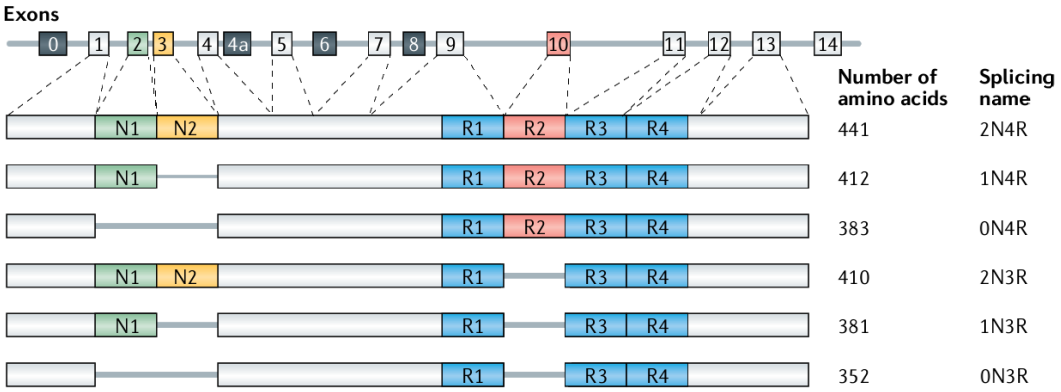

Figure 4 – Schematic representation of the human MAPT gene and the six isoforms of the human brain. (Adapted from [54]).

❖ Domains and Structure of TAU

Structurally TAU can be subdivided into two major domains: the Projection Domain (PD) and the Microtubule-Binding Domain (MiBD). The PD (N-terminal) encompasses a proline-rich region as well as an acidic region, and is so designated since it projects from the microtubule surface where it might interact with a neural plasma membrane, in addition to cytoskeletal elements such as spectrin and actin filaments, allowing for the interconnection between TAU-stabilized microtubules and neurofilaments that limit the flexibility of the microtubule grid. The PD is also important in the determination of the axonal diameter and spacing between microtubules in axons, and it is likely involved in signal transduction pathways concerning phospholipase C gamma (PLC-γ) [50–52]. On the other hand, the MiBD interacts with microtubules through some repeated domains (R1-R4) located at the C-terminal, consisting of 18 amino acids encoded by exons 9, 10, 11 and 12, separated from each other by 13- or 14- residue spacer regions [55]. The MiBD is extremely important, since it interacts with microtubules, allowing for their polymerization and stabilization, in addition to regulating their dynamic stability, being implicated in their normal function. Recent evidence also supports a role of the MiBD in the modulation of the phosphorylation state of TAU proteins, once it allows the competitive binding of microtubules to TAU, inhibiting Protein Phosphatase 2A (PP2A) activity [52].

❖ Subcellular localization and functions of TAU

The distribution of TAU, at a subcellular level is adjusted according to the developing brain [56], such that in the young neurons TAU is found among the cell body and neurites, while after the formation of axons and polarization of neurons, TAU is found in greater amounts in axons and depleted in dendrites and nuclei [57]. For this reason, the presence of TAU in dendrites represents one of the first signs of neurodegeneration, although some studies suggest that its presence in such subcellular localization might be related to a role in the regulation of synaptic plasticity [54]. This relocation of TAU might be due to several factors, namely the greater affinity of this protein to microtubules in axons than in dendrites [58] and the quick axonal transport of TAU right after its synthesis in the soma [59]. Importantly, different isoforms of TAU are found in different cellular compartments of neurons, which can mean that the protein has specific features depending on their subcellular localization. This way disturbance of the distribution of these isoforms might induce a gain of function of TAU that becomes toxic, resulting in neurodegeneration [54].

As previously stated, TAU has different functions according to its subcellular localization. Starting with axonal TAU, it is important to stabilize microtubules, leading to microtubule assembly and, more importantly, to regulate the dynamic instability of microtubules which facilitates the cytoskeleton reorganization [60]. Another feature of TAU is the regulation of axonal transport of cellular components such as mitochondria or neurotransmitter vesicles by influencing the function of dynein and kinesin, responsible for the transport of cargoes towards the cell body (minus end) and towards the axonal terminus (plus end), respectively. An example of this regulation relies on the fact that TAU has a stronger inhibitory effect on kinesin than on dynein and, for this reason, in the soma of cells overexpressing TAU there is an accumulation of cargoes such as APP, which is carried by kinesin-vesicles along axons and dendrites. Also, TAU might affect the axonal transport of other cargoes through competitive binding to kinesin [61]. In addition to this, TAU seems to be essential for axonal elongation and maturation, though such role still needs confirmation through further experiments [54]. Besides the axon, it is believed that nuclear TAU is responsible for maintaining the integrity of genomic DNA, cytoplasmic RNA and nuclear RNA [62].

Moreover, TAU may interact with actin, modifying the organization of the cytoskeleton network by inducing the alignment of bundles consisting of actin filaments [54].

❖ Post-translational modifications and implications in TAU function

In tauopathies such as AD, the typically soluble TAU is present in an abnormal filamentous form, resulting from conformational changes and misfoldings in the normal structure of TAU, ultimately leading to the appearance of intraneuronal protein aggregates, thereby preventing the correct organization of the cytoskeleton [48]. The mechanisms responsible for TAU's loss of function are not yet fully defined. However, like many proteins, TAU undergoes post-translational modifications, such as phosphorylation (hyperphosphorylation), acetylation, glycation, ubiquitination, nitration, truncation and some other modifications, which are believed to be the main cause of loss of normal function and acquisition of pathological features such as protein aggregation [63].

Regarding the phosphorylation, the longest variant of TAU (2N4R) has 85 potential phosphorylation sites (45 serines, 35 threonines and 5 tyrosines) that are phosphorylated and/or dephosphorylated by multiple kinases or phosphatases, due to their easy accessibility resulting from TAU's natively unfolded structure [54]. Most of these potential sites are clustered in regions flanking the MiBD repeats (R1-R4), namely in the proline-rich region and in the C-terminal extreme, with the exception of Ser261 (R1), Ser285 (R1-R2 inter-repeat), Ser305 (R2-R3 inter-repeat), Ser 324 (R3), Ser352 (R4) and Ser356 (R4) [52]. Since phosphorylation plays a crucial role in regulating the physiological functions of TAU, such as the binding of TAU to microtubules and ultimately their stabilization and assembly, multiple studies have focused on the protein kinases and protein phosphatases responsible for the regulation of this microtubule-associated protein [54].

The main kinases involved are the Glycogen Synthase Kinase 3β (GSK-3β), which is a serine/threonine protein kinase widely expressed in the brain and associated with microtubules [64]; and Cyclin-Dependent Kinase 5 (CDK5), which is a serine/threonine protein kinase, member of the CDK family [65]. Interestingly, CDK5-mediated phosphorylation leads to further phosphorylation of TAU by GSK-3β, favouring AD pathogenesis [66]. This could be due to conformational changes that allow the access of GSK-3β as well as other kinases to further phosphorylate TAU [48,54]. As for dephosphorylation of TAU, the phosphatases more likely involved are Protein Phosphatase 2A (PP2A), which accounts for ≈70% of the human brain TAU phosphatase activity [67], followed by and Protein Phosphatase 1 (PP1) . Studies show that the activity of PP2A is diminished in the brain of AD patients, which could result from post-translational modifications of its catalytic domain, reduced PP2A expression, as well as increased levels of endogenous PP2A inhibitors [68,69].

1.4 Genetics of AD

As previously said, AD is a complex multifactorial dementia and, although our knowledge of the pathophysiology still remains in an initial state, it is broadly accepted by the scientific community that genes play a crucial role in both onset and development of the disease [70].

To date, more than two hundred mutations have been linked to AD and every year more are discovered [10] . Depending on the genetic cause and the age-of-onset, AD can be classified as Early-Onset Alzheimer Disease (EOAD) or Late-Onset Alzheimer Disease (LOAD). The latter is the most common type of AD [10,71,72].

1.4.1 Early-Onset Alzheimer Disease (EOAD)

The EOAD tend to group within families, sometimes throughout generations [73]. Usually it develops before the age of 65 years old and is transmitted as an autosomal dominant trait, caused by extremely penetrant mutations. Only about 5% of AD cases fit in this category. To date, three genes have been highly related to this type of AD: *APP*, *PSEN1* and *PSEN2* [3,70,72] (Table 1). The first gene discovered was the APP gene, in 1987, which lies in the long arm of chromosome 21 [74]. Due to its location, patients who suffer from Down syndrome tend to develop AD later in life, as they have an extra copy of the *APP* gene[2].

The first missense mutation in APP was reported in 1991 [75], after which nearly 20 other AD mutations have been reported in this gene. Most of the *APP*-variants take place close to the recognized γ-secretase site, between residues 714 and 717, suggesting that γ-secretase cleavage of APP is critical for AD development [70]. A few years later, other mutation was linked to the development of EOAD. This mutation occurs on chromosome 14q24 and affects the *PSEN1* gene, which encodes for presenilin 1 [76], a highly conserved membrane protein necessary to release Aβ from APP during the γ-secretase action. Every year new mutations are discovered to affect this gene. Subsequently mutations in *PSEN2* (located in chromosome 1) were identified and associated with AD [70]. These three genes are all involved in the production of Aβ peptides, which gave rise to the Amyloid Hypothesis in the development of AD [72], whereby the pathophysiological process that ultimately result in the appearance of the neuropathological features of AD (SPs and NFTs), as well vascular damage and inflammation, are consequences resulting from the abnormal production of Aβ, rather than causes of the disease process [77].

Table 1 – Overview of the stablished EOAD genes and their functional relevance for AD pathogenesis. (Adapted from [72])

Gene	Chromosomal location	Mode of inheritance	Number of pathogenic mutations (affected families)[a]	Relevance for ad pathogenesis
APP	21q21.3	Autosomal-dominant	51 (121)	Increase in Aβ (Aβ42/Aβ40 ratio); mutations close to γ-secretase site
PSEN1	14q24.3	Autosomal-dominant	219 (480)	Increase in Aβ (Aβ42/Aβ40 ratio); essential for γ-secretase activity
PSEN2	1q42.13	Autosomal-dominant	16 (34)	Increase in Aβ (Aβ42/Aβ40 ratio); essential for γ-secretase activity

[a](Source: "AD & FTD Mutation Database" [URL: http://www.molgen.ua.ac.be/ADMutations/] current on 6/10/2016)

1.4.2 Late-Onset Alzheimer Disease (LOAD)

Most cases of AD are Late-Onset, representing about 95% of all cases [3], affecting people with 65 or more years of age. This type of AD is much more complex and less understood, both genetically and epigenetically, with the possible involvement of several genes. There can also be gene-gene and gene-environment interactions, which makes its appearance more unpredictable as the genes involved may contribute to or prevent the risk of developing the dementia, without causing it directly [71,73].

Until recently only one gene had been associated with this form of AD. The gene encoding apolipoprotein E (*APOE*), located in chromosome 19, was linked to LOAD in 1993, after Aβ was found to bind APOE [78]. This association is due to the ε4 allele of *APOE*, which has been consistently proved to be a risk factor of LOAD [3,72]. However, studies have shown that *APOE* is limited to account for approximately 50% or less of LOAD risk [79], suggesting that other genes, and therefore other hazard loci, could contribute to LOAD risk .

In order to identify the remaining genes for LOAD, two methods have been suggested, namely Whole Exome Sequence (WES) and Genome-Wide Association Studies (GWAS) [19,80]. The latter, allowed to overcome several technical limitations, due to the advent of microarray technology which makes it possible to evaluate several hundreds of thousands of Single-Nucleotide Polymorphisms (SNPs) in a single trial. Nevertheless, despite having several advantages once it is an approach hypothesis free, GWAS require more rigorous criteria to validate the significance of SNPs in a disease context [10]. Also, since the effect sizes of the novel genes possibly related to AD are small, it requires a remarkably big number of individuals (cases and controls) in order to identify additional genes involved in LOAD, which represents another disadvantage of this method [81].

Since 2009, and in addition to APOE, more than 20 loci have been identified by five GWAS and one meta-analysis, and significantly associated to LOAD. Among these are *CLU, CR1, PICALM, BIN1, ABCA7, MEF2C, HLA-DRB1/HLA-DRB5, EPHA1, CD2AP, CD33, INPP5D, NME8, ZCWPW1, PTK2B, SORL1, CELF1, SLC24A4/RIN3, FERMT2 and CASS4* [19,82–85]. Depending on the possible role in the process of AD, these genes cluster in a specific set of pathways, namely Lipid Metabolism, Immune Response or else Endocytosis, strongly highlighting the relevance of these molecular mechanisms in AD aetiology [81,86]. Of all these genes, and according to the Alzgene database (http://www.alzgene.org/), the bridging integrator 1 (*BIN1*) is currently recognised as the most significant LOAD-associated risk loci after APOE, and will be discussed in more detail in section 1.5.

Despite all these advances and discoveries allowed by GWAS, a significant part of the genetic contribution to AD has not yet been explained and the APOE-ε4 allele remains the key gene variant considered to be a LOAD risk factor.

1.4.2.1 APOE as a genetic risk factor for AD

Apolipoprotein E (APOE) is a lipoprotein of 299 amino acids and 34kDa molecular weight, first discovered in 1970s as a protein component of triglyceride-rich lipoproteins [87].

This protein has three major isoforms APOE2, APOE3 and APOE4 encoded by three alleles located on a single gene locus on position 19q13, being them ε2, ε3 and ε4, respectively [88]. The isoforms differ by a single-amino acid substitution at residues 112 and 158 (Figure 5), which provides structural changes that influence their functions both at a cellular and molecular level, allowing the association of different isoforms to different neuropathological conditions [89].

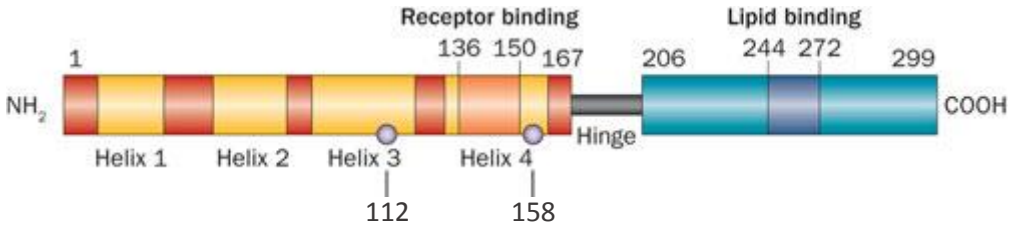

Figure 5 – Schematic representation of the structure of APOE. The different isoforms APOE2, APOE3 and APOE4 differ from one another at amino acid residues 112 and 158 (grey circles) providing unique features to each isoform. (Adapted from [90]).

Therefore, APOE3, which is the most common of the three isoforms and considered the normal form of APOE, has Cys-112 and Arg-158 [91]. On the other hand, APOE2 has Cys-112 and Cys-158, the latter being responsible for greater stability of this isoform and associated with a protective effect against AD [92]. Finally, the APOE4 which has Arg-112 and Arg-158, is a major risk factor for AD since the presence of one APOE-ε4 allele increases the risk to develop the dementia by about three times, while having two copies of the allele increases LOAD risk by 12 times [93] (Table 2). Furthermore, APOE-ε4 allele shifts the age of the disease onset, with each allele lowering the age of onset by one or two decades relative to non-carriers in LOAD [94].

Importantly, while in EOAD mutations in the APP gene, the PSEN 1 gene or in the PSEN2 gene are sufficient, but not required, to cause AD, the APOE-ε4 allele is neither indispensable nor necessary to cause AD, being considered a risk factor that diminishes the age of onset, as previously said [95].

Table 2 – Comparison between APOE isoforms. Isoform-specific amino acid difference and allele frequency in each isoform for the general population and patients with Alzheimer's Disease. (From [90])

	Isoform-specific amino acid difference		Allele frequency (%)	
	112	**158**	**General**	**AD**
APOE2	Cys	Cys	8.4	3.9
APOE3	Cys	Arg	77.9	59.4
APOE4	Arg	Arg	13.7	36.7

Functionally, APOE usually is important for lipid metabolism as well as transport, and is a protein expressed in many organs, mainly in the liver followed by the brain. APOE occurs primarily as a constituent of lipoprotein complexes alongside with other apolipoproteins and proteins in plasma as well as cerebrospinal fluid (CSF) [96]. In peripheral tissues, APOE is primarily produced by the liver and macrophages, being secreted into the circulation as a protein incorporated into very low-density lipoproteins (VLDL), chylomicron remnants, and certain subclasses of high-density lipoprotein (HDL) [86,89]. APOE plays an essential role in the regulation of cholesterol as well as lipids throughout the organism facilitating the clearance of plasma lipoproteins through the low-density lipoprotein receptor (LDLR) as well as additional LDLR-related protein family members [87]. In addition, in the central nervous system (CNS), this lipoprotein produced primarily by astrocytes [97,98], may be involved in several physiological and pathological processes, including the metabolism and trafficking of cholesterol, resulting from neurodegeneration, to neurons lacking them for membrane repair, proliferation or remyelinization [86]. Despite neuronal trafficking, APOE also plays an essential role in synaptogenesis, blood-brain barrier integrity [97,98], and it affects glutamate receptor function and synaptic plasticity by modulating neuronal APOE receptor recycling [99].

Although the mechanism by which APOE isoforms affect the risk to develop AD is not completely understood, significant evidence of diverse neuropathological effects of APOE4 on cells within the CNS have been validated. Decreased Aβ clearance [89] and increased Aβ aggregation [100,101] has been demonstrated in ε4 carriers, as well as Aβ load and plaque accumulation strongly correlated to APOE-ε4 dosage at autopsy [102,103]. Finally, enhanced formation of C-terminal-truncated fragments characteristic of ε4 isoform stimulate TAU phosphorylation leading to preneurofibrillary tangles [104].

It is important to keep in mind the fact that APOE has different influences in AD onset and progress according to the population ethnicity [105,106] and so it is extremely important to understand the relevance of this lipoprotein to each type of population since it might be useful for diagnosis purposes.

1.5 Bridging Integrator 1 (*BIN1*)

BIN1, also named Amphiphysin2, is a member of the *BIN1*/amphiphysin/RVS167 (BAR) family of genes, encoding a nucleocytoplasmatic adaptor protein highly expressed in the CNS. *BIN1* maps to the long arm of the human chromosome 2 (2q14.3) and encodes no less than 20 exons [107], which can be spliced into several isoforms. Primarily, 19 exons were identified [108], after which an extra exon was discovered between exons 6 and 7, named exon 6a [109].

1.5.1 BIN1 domains and isoforms

In mammals BIN1 is widely expressed, but since *BIN1* transcripts undergo an extensive differential splicing, more than 10 isoforms are produced with different subcellular localization, tissue distribution, and particular functions according to specific protein interactions. Furthermore, depending on the inclusion of four exons (6a, 10, 12 and 13) different types of isoforms are produced, including brain specific isoforms 1-7, muscle-specific isoform 8 and isoforms 9 and 10 (ubiquitously expressed) [109]. Also an aberrant isoform has been reported to be expressed specifically in tumour cells [108]. Depending on the isoform expressed, *BIN1* encodes proteins ranging from 409 amino acids (isoform 10) to 593 amino acids (isoform 1), having a predicted molecular weight of 45 kDa to 65 kDa, respectively.

Relatively to BIN1 structure, it has numerous characteristic protein domains [110]. The BAR domain (N-terminal) is ubiquitously expressed and binds lipid membranes in a dimer conformation, stimulating membrane curvature in places such as T-tubules in muscular cells, endocytic pits in neuronal and non-neuronal cells, and possibly in cytoplasmic endosomes [111,112]. Importantly, the protein-protein interaction with dynamin can be modulated through differential splicing of exon 7 in the BAR domain [113]. In some brain isoforms there is also a N-terminal insert domain of 31 residues, encoded by exon 6a, located within a putative coiled-coil region in the BAR domain [114].

Another ubiquitously expressed domain is the Src Homology 3 (SH3) domain, which is encoded by exons 19 and 20 and allows the binding of proline-rich motifs [115]. Interestingly, this domain in BIN1 differ from other SH3 domains owing to a large patch of negative electrostatic potential and a remarkably prolonged n-Src loop [116]. The Myc-binding domain (MBD) is present in all isoforms, being encoded by exons 17 and 18 [117], though alternative splicing of the first might lead to the loss of the interaction between BIN1 and c-Myc [108,118]. Furthermore, exon 13 differentially spliced in a tissue-independent manner, encodes part of the MBD [109]. Between BAR

and MBD, in the muscle-specific isoform (isoform 8) exon 10 translates into a small domain of 15 residues which includes a putative nuclear localization sequence (NLS), as well as a lipid-binding sequence [117,119]. Finally, in brain-specific isoforms there is a clathrin and AP2 (CLAP) binding domain, which is encoded by exon 12 (including a series of alternative brain-specific exons 12A-D) and is responsible for binding to endocytic proteins, namely clathrin and AP2/α [120,121] (Figure 6).

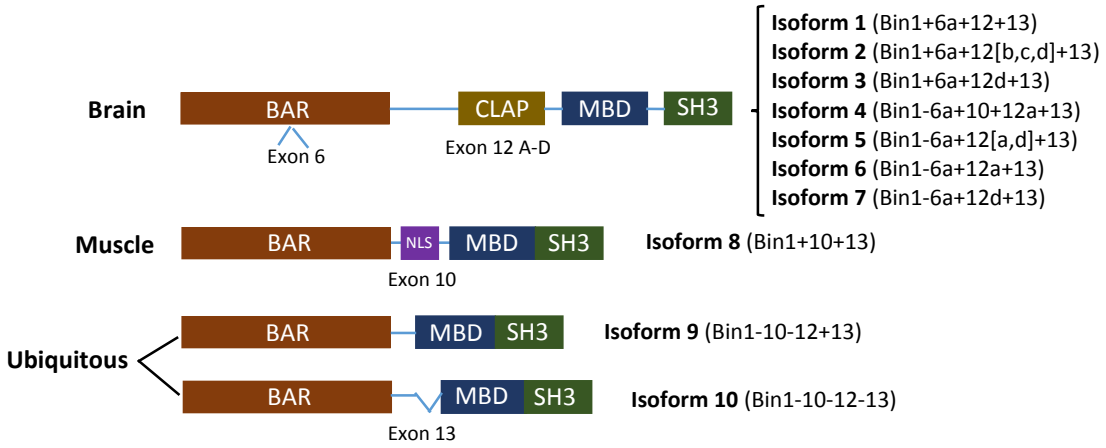

Figure 6 – Schematic representation of BIN1 domains and isoforms. Exons 6, 10, 12 (a-d) and 13 are alternatively spliced generating brain specific isoforms (1-7), a muscle isoform 8 and the two ubiquitously expressed isoforms (9 and 10). Abbreviations: BAR, BIN1/Amphiphysin/RVS167 domain; CLAP, clathrin-AP2 binding region; MBD, Myc-binding domain; SH3, Src homology domain; NLS, nuclear localization sequence. (Adapted from [109])

1.5.2 BIN1 cellular functions

The cellular functions performed by BIN1 are regulated through specific exon splicing, thus each isoform has a particular role in the organism, also depending on subcellular localization and tissue distribution. Furthermore, BIN1 may also be regulated by some post-translational modifications such as protein phosphorylation. Additionally, epigenetic processes, such as DNA methylation, might result in the inactivation and silencing of *BIN1* by affecting the promoter activity, since it contains a CpG island [108].

Several studies have associated BIN1 to the process of clathrin-mediated endocytosis as well as intracellular endosome trafficking both in neuronal and non-neuronal cells [107,122,123], through the interaction with numerous proteins, namely dynamin [122], AP2 adaptor complexes [122,123], clathrin [121], synaptojanin [124], and endophilin [125], reflecting its ability to regulate membrane remodelling.

Furthermore, BIN1 has a potential role in regulating the actin cytoskeleton, coordinating membrane and cytoskeleton remodelling, given that it appears to link the two via the tubular membrane structure it forms. Thus, BIN1 might be involved in some AD neuropathological features

such as NFTs, since it might bind to the coiled-coil region of a plus-end protein involved in microtubules stability (CLIP170) though its BAR domain [110,126]. Finally, BIN1 has numerous nuclear functions and has been associated to DNA repair, cell cycle and apoptosis [110]. In fact, the MBD of BIN1 interacts with c-myelocytomatosis (c-MYC) oncoprotein, which has a crucial role in cell growth, apoptosis as well as malignancy [117], and several studies have shown that blocking BIN1 expression or else BIN1-Myc interaction resulted in specific inhibition of Myc-mediated apoptosis [127,128] . It is important to note that only nuclear isoforms of BIN1 can activate apoptosis. Relative to DNA repair, BIN1, through the binding of its BAR domain to proteins which have a major role in this process, can modulate their activity [110].

1.5.3 *BIN1* in AD

Since AD is such a multifactorial disease, recently several GWAS have been performed in order to identify risk loci involved in the pathogenesis of LOAD.

After several studies, *BIN1* has repeatedly been confirmed as a significant LOAD-associated risk loci, occupying the second position just after APOE [83,129–131]. However, this locus was only considered statistical significant after the study performed by Seshadri *et al*. [129],being subsequently confirmed in other studies. From all the *BIN1* Single Nucleotide Polymorphisms (SNPs) analysed, two were especially relevant for AD, being them rs7561528 and rs744373, both of which lie approximately 25kb to 30 kb upstream of the *BIN1* gene, respectively. The first one has a risk allele "G", while the second one has a risk allele "A" [109]. Importantly, this work will be focusing on the SNP rs744373 since it has been correlated with the rate of cognitive decline and AD progression, in addition to its risk allele "G" being linked to faster Mini-Mental State Examination (MMSE) deterioration, though this outcome does not reach statistical significance [109].

The significance of *BIN1* as a risk loci to AD have been successfully replicated and confirmed in several independent candidate gene studies evaluating ethnically distinct populations, such as Caucasian and Caribbean Hispanic [132,133]. Furthermore, the significance of *BIN1* as a genetic risk locus to LOAD has been replicated and confirmed in the largest family-based GWAS [134], as well as in African American populations [135]. However, this association was not detected in the Han Chinese [136] nor the Korean population [137]. Importantly, the most significant SNPs may vary from study to study, according to the datasets consisting of different ethnicities. [109] Besides, in a population, more than one SNP may be associated to AD, therefore more GWAS should be performed in order to

characterize, the best way possible, which SNPs contribute to AD in different populations. In addition, it is possible that the various SNP's of *BIN1* differentially affect the development of AD and, if this is true, it would be extremely interesting and useful to split subject groups and investigate the degree of cognitive decline separately for each *BIN1* genotype [109].

Some studies have analysed the expression of *BIN1* in the human brain, detecting an increase of *BIN1* transcripts in brains of AD cases when compared to controls [138]. However, there are also studies that reveal a significant decrease of BIN1 in the brain of LOAD patients (87% when compared to non-demented age-matched controls) [139]. In addition, although several SNPs of *BIN1* have been associated to LOAD, little is known about BIN1 protein expression and its contribution to AD pathogenesis. Thus it is imperative that more studies are performed in order to help clarify the role of *BIN1* in AD pathogenesis, identifying in which pathways that genetic locus may be involved, and in which way that involvement contributes to the onset of AD or its progression.

Since BIN1 has a role in the process of clathrin-mediated endocytosis, some studies have focused on the potential role of this BAR protein in APP metabolism and Aβ production, since they are both internalized through the endolysosomal trafficking pathway. Following this hypothesis, fluctuations on BIN1 expression would result in changes in APP trafficking through intracellular compartments, influencing whether APP undergoes the non-amyloidogenic or the amyloidogenic pathway, depending on the localization of the cleaving secretases. Thus, having been cleaved by β-secretase in the endosomes, if APP moved into the cell surface it would be cleaved by α-secretase, however, if APP moved into lysosomes it would be cleaved by γ-secretase, giving raise to Aβ [140,141]. Despite this, recent studies tried to investigate the importance of *BIN1* in Aβ processing and secretion but failed to find any association between the levels of BIN1 and Aβ42 neurotoxicity [138]. Also, another approach using siRNA to knockdown endogenous *BIN1*, and overexpressing *BIN1* in SH-SY5Y cells failed to have an impact on APP processing [139].

As mentioned previously, BIN1 has a potential role in regulating the actin cytoskeleton, coordinating membrane and cytoskeleton remodelling. Thus, it may interact with microtubule-associated proteins, like TAU. To investigate this theory a recent study was performed and identified an association between *BIN1* and TAU at a biochemical, genetic and neuropathological level, strongly suggesting that increased levels of BIN1 lead to an augmented AD risk by interacting with the TAU pathway, although the exact mechanism is still not clear. Some of the possibilities lie on the fact that previous studies show that BIN1 can stabilize T-tubule structure in muscles. Thus, BIN1 might modulate microtubule stability or else TAU phosphorylation/aggregation [138]. Also, BIN1

might be involved in NFTs formation. However, although BIN1 and TAU colocalize, there has not been detected any colocalization between BIN1 and NFTs. This suggests that BIN1 can influence the early stages of AD, being a primary contributor to the disease through the promotion of TAU aggregation, rather than influencing the late stages of the TAU-related pathology when NFTs are formed. In addition, the knockdown of *BIN1* resulted in the suppression of TAU-induced neurotoxicity [138]. Despite these findings, a study had some contrasting results, showing no correlation between BIN1 and the amount of TAU pathology in AD [139]. For this reason, more studies should be performed in other populations, in order to investigate the significance of *BIN1* in AD pathogenesis, potentially through the TAU pathway.

1.5.4 *BIN1* and other diseases

Besides AD, *BIN1* has been associated to other diseases such as cancer, myopathies, namely centronuclear myopathy (CNM) and myotonic dystrophy (MD), and has also been linked to cardiac failure, though the latter requires more in-depth investigation [110].

Starting with cancer, as stated above, BIN1 interacts with c-MYC and so it can inhibit MYC-dependent transformation and tumour growth, as long as it is expressed at a standard level [117]. Also, several studies have shown that the expression of BIN1 is reduced or altered in numerous cancer types, such as colon, prostate, breast and lung cancers, as well as hepatocarcinoma and neuroblastoma [142–145]. Furthermore, a study was performed, and revealed that the decrease of BIN1 expression leads to an increase of resistance to Cisplatin-based chemotherapy, which may be due to loss of control over the DNA repair mechanism [146]. Therefore, augmenting the expression of BIN1 might be an unusual and interesting strategy for the treatment of cisplatin-resistant cancers. Also, since *BIN1* has been linked to cytoskeleton and membrane remodelling, it could be implicated in tumour cell migration and invasion, whereby its decrease would contribute to tumour progression.

BIN1 is greatly expressed in the brain and skeletal muscles. In the latter, several studies have shown that BIN1 has a major physiological relevance, since the increase of its expression leads to myoblast fusion and differentiation, being also essential for the positioning and remodelling of T-tubules. This BAR protein might also be responsible for sarcomere organization, once again highlighting its importance to skeletal muscle [110]. Therefore, changes in BIN1 expression might result in diseases related to this type of tissue.

1.6 Diagnosis of AD

The assessment of AD made by an individual's primary care physician or neurologist, requires not only the presence of memory decline but also multiple cognitive deficits. The diagnosis criteria and guidelines for AD were first established in 1984 by the Alzheimer's Association and the National Institute of Neurological Disorders and Stroke (AANINDS) [147] and last updated almost three decades later, in 2011, by the National Institute on Aging – Alzheimer's Association (NIA-AA) [148].

Usually the physician starts with the medical and family history of the patient, plus psychiatric history as well as behavioural, personality and cognitive changes. During this stage of the diagnosis it is extremely important to interview a family member or a friend close to the patient, since they may be useful to detect that something is wrong even before changes are evident on tests, and can even explain how cognitive abilities, practical skills and behaviours have changed over time [149]. After the first assessment, the physician also performs neuropsychological tests, such as the Mini-mental state exam (MMSE), which allows the measurement of general cognitive function, with scores ranging from 0 (severe impairment) to 30 (no impairment), thus evaluating everyday mental skills.

Due to the progress in radiological imaging techniques, Magnetic Resonance Imaging (MRI) and other neuroimaging techniques such as Computerized Tomography (CT) and Positron Emission Tomography (PET), these are being widely used for AD diagnosis [3]. MRI exams are routinely requested, since they are three-dimensional, non-invasive and allow the characterization of the brain's structure, just in a few minutes. There are several types of MRI, and some of the things that were possible to study using this technique go through the assessment of the integrity of white matter fiber tracts [150], to the study of local patterns of brain atrophy in individuals suffering from MCI and AD [30,151,152], and lastly the measurement of intrinsic brain activity, which takes place without any external stimulation, revealing changes in neuronal network activities in AD patients or individuals at risk for developing the dementia [153,154]. MRI is very useful since it helps to rule out normal pressure hydrocephalus, brain tumours, cerebral hematomas or even cerebrovascular lesions [3]. Another plus of this technique relies on the fact that it allows the detection of brain abnormalities in individuals who might be at risk to develop AD but have no apparent symptoms [155].

Like MRI, PET has proven to be useful in the diagnosis of AD. Using 18F-fluorodeoxyglucose (FDG-PET) it was possible to differentiate between different forms of dementia, mainly AD and Frontotemporal Dementia (FTD), through the measurement of local brain metabolism [156,157]. Recent advances certified the use of a radioactive compound named Pittsburgh compound B (PiB) during PET assessment of AD, since it binds specifically to amyloid plaques in the brain. That way it is possible to detect one of the neuropathological hallmarks of AD, in a non-invasive way. However, there are some concerns about the use of PET-based amyloid imaging in the clinical setting, notably due to its high cost and low positive predictive value [158].

Another way of diagnosis that has been widely studied are the CSF-based protein biomarkers. Through these studies it was possible to discover that the levels of Aβ42 are reduced in AD patients, while the levels of phosphorylated TAU (P-TAU) are higher [159,160]. Plasma biomarkers have also been suggested as an option to CSF biomarkers [161].

Despite this, since AD is such a complex disease, innovative methods for AD biomarker identification as well as validation need to be established, in order to promote early AD diagnosis and therapeutic interventions [162].

1.7 Treatment of AD

Currently there is no cure for AD and scientists are just beginning to discover therapeutic methods that aid handling the symptoms of the dementia but do not delay the evolution or reverse the progress of the disease itself [163].

Therapeutically AD presents no great difference from other dementias, using drugs based on transmitter-replacement therapies. Given that, in AD, as the initial affected neurons are the glutamatergic and acetylcholinergic, the first therapeutic approach is to administer Acetylcolinesterase inhibitors that will promote cholinergic neurotransmission by preventing the breakdown of acetylcholine, thus improving memory function and attention in AD patients. Currently there are three cholinesterase inhibitors approved by U.S. Food and Drug Administration (FDA). These are galantemine, donezepil and rivastigmine. Another FDA approved drug is memantine, an NMDA (glutamate) antagonist whose function is to prevent excessive glutaminergic transmission that may result in excitotoxicity because of high intracellular calcium concentration in circumstances of overstimulation [164].

Currently, growing evidences support the amyloidogenic hypothesis leading scientists to seek new therapeutic approaches in order to decrease the amount of Aβ in the brain. One class of such drugs are secretase inhibitors or modulators, thus preventing the processing of APP and production of Aβ [165]. An alternative method is the use of inhibitors of amyloid aggregation or drugs that promote the clearance of Aβ. It is expected that this type of drugs is able to retard the progress of AD [166].

Furthermore, as knowledge about the pathological mechanism underlying AD improves, additional therapeutic approaches are being studied namely drugs that target the abnormal TAU protein that originates the NFTs [167].

Concerning the behavioural and emotional changes, typical in AD patients, there are some pharmacological options including antipsycotics, antidepressants or even anxiolytics, and some non-pharmacological approaches such as relaxation and physical exercise that should not be ruled out to help promote a better quality of life for the patient [164].

2. AIMS OF THE THESIS

AD is the most common form of age-related dementia, being a complex multifactorial disorder in which genes play a crucial role in both onset and development of the disease.

Most cases of AD are late-onset and, until recently, only the APOE gene had been associated to it. However, in the last few years through GWAS, *BIN1* has been recognised as the most significant LOAD-associated risk loci after APOE, encoding a nucleocytoplasmatic adaptor protein highly expressed in the CNS.

Although several SNPs of *BIN1* have been associated to LOAD, two are especially relevant for AD, including rs744373. However, little is known about BIN1 protein expression and its contribution to AD pathogenesis. Thus, it becomes imperative to perform more studies in order to help clarify the role of *BIN1* in AD pathogenesis. In addition, the most significant SNPs may vary from study to study, according to the datasets consisting of different ethnicities, whereby it is important to characterize, the best way possible, which SNPs might contribute to AD in different populations.

The main goal of this thesis was to address if *BIN1* polymorphism rs744373 can be considered a SNP that confers risk to the development of AD in a Portuguese population, specifically in the Aveiro district. Therefore, the particular aims of this work were to:

- Amplify the polymorphic region of *BIN1* gene (rs744373) by Polymerase Chain Reaction (PCR);
- Determine the genotype of the study population through Sanger sequencing;
- Determine the *BIN1* (rs744373) genotypic and allelic frequencies in the pilot study group;
- Evaluate the statistical significance of rs744373 as a risk factor to develop AD, in the study population;
- Study the association of rs744373 with APOE-Ɛ4 carriers and CDR;
- Study the association of rs744373 with Diabetes Mellitus.

3. MATERIALS AND METHODS

3.1 Study Group

The pilot study group was chosen from a cross-sectional population-based survey carried out at the University of Aveiro, which had the participation of 590 individuals from the Aveiro district. This set of individuals belonged to a primary cared based group (pcb-Cohort), of which 568 were selected since they fulfilled the inclusion criteria (individuals aged 50 years or more). These 568 individuals were assessed for their cognitive abilities, as well as the level of dementia. Thus, screening tests included Clinical Dementia Rate (CDR), Mini Mental State Examination (MMSE), Geriatric Depression Scale (GDS), Katz of Activities of Daily Living (ADL) and Instrumental Activities of Daily Living (IADL). All of these test were previously standardized for the Portuguese population, with the exception of ADL test [1].

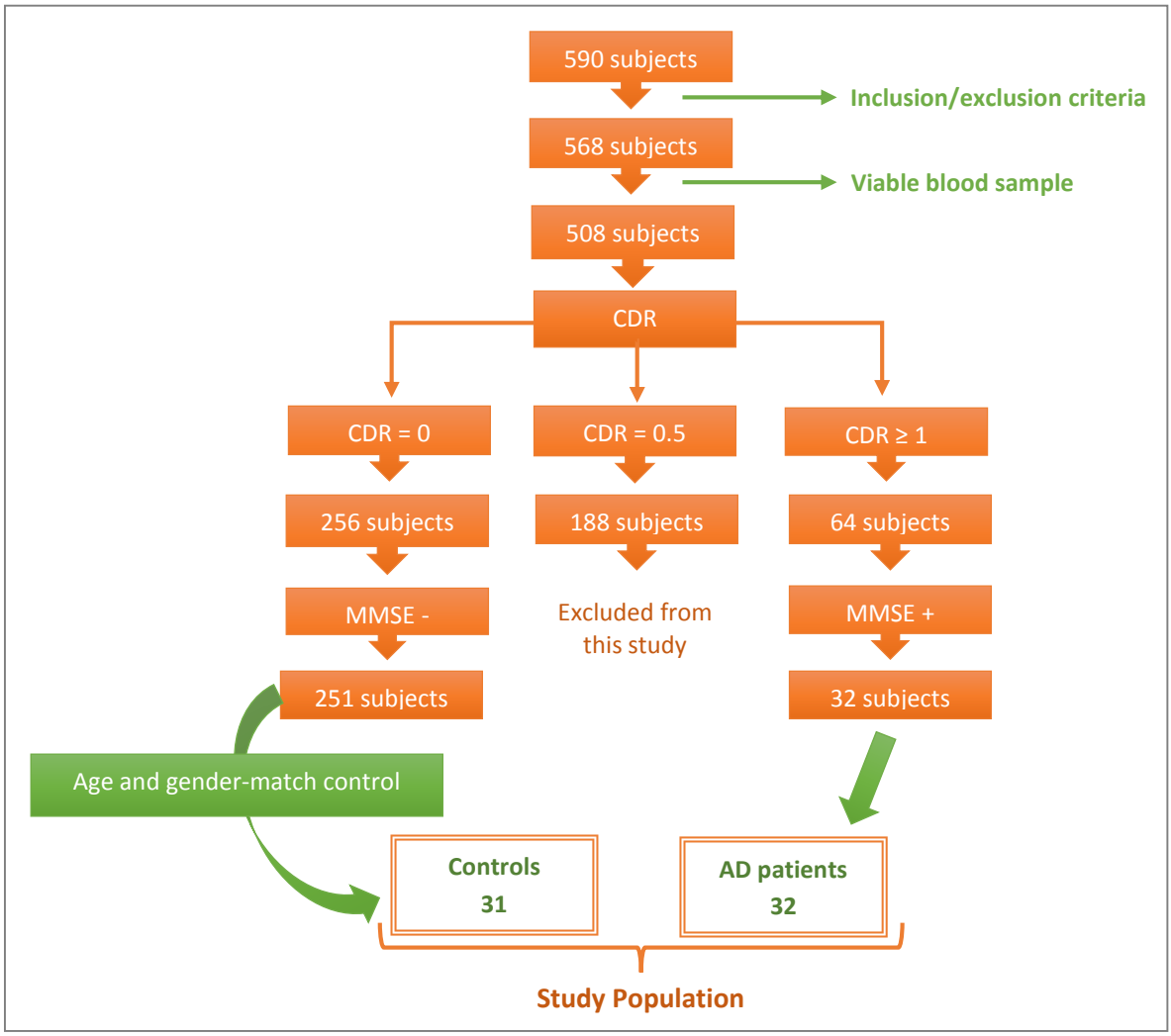

Figure 7 – Schematic diagram of the Study Design. A pilot study of 63 individuals (32 AD patients and 31 Controls) was chosen for the work here described.

Of the 568 individuals that fulfilled the inclusion criteria, 60 were excluded for this study because they didn't have a viable blood sample, leaving 508 individuals. According to the cognitive evaluation and dementia screening performed in these individuals, for the present study we selected the ones which were CDR≥1 (64 individuals) and had a MMSE test positive for cognitive deficit, resulting in a total of 32 individuals considered as potential AD patients. Accordingly, 32 individuals selected as AD patients, and 31 healthy control subjects (CDR = 0, and MMSE showing no cognitive deficits) matched for gender and age were chosen. The study design can be visualized in Figure 7.

In summary, the study population consisted of 63 individuals, of which 31 were Controls and 32 potential AD patients. The latter group of individuals is herein referred to as AD patients.

3.2 Ethical Approval

The study from which AD patients and Controls were selected for the present work, was part of a project approved by the ethics committee for health of the Central Regional Administration in Coimbra (ARS) (Comissão de Ética para a Saúde da ARS Centro, protocol number 012 804 of April 4, 2012), and by the National Committee for Data Protection (Comissão Nacional de Protecção de Dados).

Also, confidentiality of the data collected of each individual was assured.

3.3 Sample Collection

Blood samples were collected in 3 tubes (3mL for whole blood, 5mL for serum and 5mL for plasma) from each participant upon arrival, and placed in an EDTA tube, according to standard procedures, in order to prevent coagulation. As soon as the samples arrived at the laboratory, these were aliquoted right away and frozen at -80°C, being stored at iBiMED's biobank.

It is important to note that, despite having 568 individuals fulfilling the inclusion/exclusion criteria, only 508 blood samples were available. This was due to the fact that some individuals were quite old, making it difficult to collect blood samples. It was decided not to subject them to the procedure, in order not to cause any discomfort or pain to the volunteer.

3.4 *BIN1* Genotyping

BIN1 genotyping was performed resorting to the use of Polymerase Chain Reaction (PCR) technique, in order to amplify the selected SNP variant rs744373. Subsequent to amplification, PCR products were precipitated. In order to attest the successful amplification of the DNA fragments, samples were analysed through an agarose gel electrophoresis.

3.4.1 Amplification of *BIN1* gene (rs744373) by PCR

PCR is a technique used to create multiple copies of a particular DNA sequence, using a DNA template. This way it is possible to yield enough DNA required for analysis. This technique was used to amplify the SNP rs744373 of *BIN1* gene from the genomic DNA (gDNA) of each patient.

There was no prior DNA extraction, since it was done directly by using Phusion Blood Direct PCR Master Mix (ThermoFisher Scientific, USA), according to the manufacturer's instructions.

Primers Forward (Fw) and Reverse (Rv) were design according to the scientific report of Darawi *et al*.[168] and are shown in Table 3.

Table 3 - Sequence of Primers Forward and Reverse for the genotyping of the polymorphism od *BIN1*.

Gene	SNP ID	Primer Name	Sequence (5'-3')
BIN1	rs744373	*BIN1*-Fw	AAG ACG GAG AGA GGA GGC AT
		BIN1-Rv	CCA TCT TCT TCT GCT CTC CCA G

Each PCR reaction was executed in a DNase free PCR microtube and the final volume of all PCR products was 20µL. The components of the reaction were added according to the order they are presented in Table 4.

Table 4 - Components of the mixture used for PCR.

Component of the Reaction	Volume/Reaction (µL)
Ultrapure H_2O (DNase/RNase-free)	7µL
Primer *BIN1*-Fw	1µL
Primer *BIN1*-Rv	1µL
2XPhusion Blood II DNA Polymerase Master Mix	10µL
DNA template (sample)	1µL
TOTAL	**20µL**

The PCR technique was performed resorting to the use of thermocycler Eppendorf® Mastercycler (Sigma-Aldrich, USA). Cycle conditions for DNA amplification are indicated in Table 5.

Table 5 - Cycle conditions for the amplification of *BIN1* polymorphism.

Number of Cycles	Temperature	Time	Stage of PCR
1	98°C	5 min	Initial denaturation
35	94°C	1 min	Denaturation
	63°C	30 sec	Annealing
	72°C	46sec	Polymerization (extension)
1	72°C	5 min	Final extension
-	4°C	∞	-

After DNA amplification, PCR products were centrifuged with Spectrafuge Mini Centrifuge (Labnet International, USA) at 1000×g (about 4000rpm) during 1 to 3 minutes in order to sediment all blood residues. Following this step, samples were precipitated.

3.4.2 Precipitation of PCR products

After PCR products were centrifuged, 10µL of the supernadant were removed and placed in a new Eppendorf tube (1,5mL). All microtubes were previously identified with the sample number. In order to start the precipitation procedure 1µL of Sodium Acetate (3M, pH 5.2) was added to the solution, followed by 25µL of Ethanol 100%. Samples were stored at -20°C overnight.

Afterwards, the samples were centrifuged at 14000rpm for 20minutes at 4°C. The supernatant was removed and 100µL of ethanol 70% were added to the remaining *pellet*. The samples were stored at -20°C for 20minutes, and later centrifuged at 14000rpm during 5minutes. The supernatant was removed, and the samples were then dried out completely at 37°C. Lastly, 13µL of ultrapure H_2O (DNase/RNase-free) was added to each tube in order to dissolve the remaining *pellet*. The purified DNA fragments were then kept at -20°C for long term storage and/or at 4°C for short term storage.

3.4.3 Agarose Gel Electrophoresis

Agarose gel electrophoresis is a technique used to separate DNA fragments of different sizes. Depending on the concentration of agarose present in the gel it is possible to obtain larger

pores (less agarose) or smaller pores (more agarose). Generally, gels with higher concentrations of agarose (smaller pores) are used when the objective is to separate smaller fragments of DNA. Given that DNA is a negatively charged molecule, it migrates from cathode to anode by the porous matrix of agarose, through an electric field. The migration of nucleic acids occurs according to the fragment size, resulting in a faster and further migration by the shorter molecules. The concentration of agarose present on the gel needs to be carefully chosen, in order to allow the detection of the DNA band size of interest.

Subsequently to precipitation of the PCR products, an agarose gel electrophoresis was executed in order to detect the specific band for rs744373 (767 bp). Thus, 130 mL of 1.5% agarose gels were prepared in 1x TAE buffer. The solution, heated until the agarose melted, was cooled down and 8µL of GreenSafe Premium® (Nzytech, Portugal) were added. The mixture was transferred into the gel tray and left to set for 25-35 min. For the gel run, the horizontal electrophoresis tank was filled with 1x TAE buffer. In order to prepare the DNA samples to be loaded, aliquots with 2µL gDNA of each sample were made, to which 0.4 µL of loading buffer (LB) was added to increase sample density and to add colour to the sample, facilitating loading and allowing to track the migration of samples. A 1Kb Plus DNA Ladder (ThermoFisher Scientific, USA) was used as a molecular weight marker. Gels run at 100V for 35 minutes and the purified DNA fragments were visualized under UV light and further analysed using the ImageLab 5.2.1 (Bio-Rad Laboratories, Inc., USA) software.

3.4.4 Sequencing of DNA samples

Sanger sequencing is a method of DNA sequencing, widely used for smaller-scale projects, based on the selective incorporation of chain-terminating dideoxynucleotides (analogues of nucleotide bases) by DNA polymerase, thus chain elongation is terminated selectively at A, G, C or T. Since these chain-terminating nucleotides lack 3'-hydroxyl group, further elongation of the chain is prevented. After ensuring the amplification of the DNA of interest (*BIN1* polymorphism rs744373) by observing an agarose band with 767bp on gels, a 96-well plate was prepared in order to send for sequencing. To each well 10µL of sample were added, prior to 3µL of Primer *BIN1*-Rv. Afterwards the 96-well plate was sent to STABVIDA Sequencing Service (Portugal) for direct sequencing by the Sanger method. After obtaining the sequencing results from STABVIDA these were analysed in order to determine the nucleotide polymorphisms and the respective *BIN1* genotype for each study individual.

3.4.5 Data Analysis

Differences in gender and age as well as differences in genotype frequencies between AD patients and Controls were described using Pearson's chi-square test and Cramer's V for effect size. Differences in Minor Allele frequencies between AD Patients and Controls were assessed using Pearson's chi-square test.

The strength of association between rs744373 and AD was estimated with the odds ratio (OR) with 95% confidence interval (CI). The association between rs744373 and APOE or CDR was tested through Pearson's chi-square test. An OR based on a bivariate analysis was performed to assess the association between rs744373 and Diabetes Mellitus, recurring to Pearson's chi-square. P-values were calculated two-tailed and p-value<0.05 was defined as statistical significance.

Statistical analysis was performed using SPSS 22.0 software (SPSS Inc; Chicago, IL).

4. RESULTS

4.1 Assessment of the balance between the two study groups

The main goal of this work was to evaluate if *BIN1* polymorphism rs744373 can be considered a SNP that confers risk to the development of AD in a group of 63 Portuguese individuals belonging to a population from a cross-sectional population-based study, in the Aveiro district.

Since AD is a disease strongly associated with age and gender, usually affecting individuals older than 65 and female, an important aspect to take into account in such a study is to have a population that is balanced. Thus, we verified if both the AD patients group and the Controls group were similar for age and gender in order to avoid results that could be biased due to these two factors.

In order to accomplish this, the first step was to run a statistical test to calculate the p-value and effect size (Cramer's V) for both gender and age of the two study groups (Table 6). With regard to gender, the number of men is the same in both groups and the number of women only differ in one individual, having a p-value of 0,932 and an effect size of 0,011. Regarding the age, when comparing the group of AD patients and the group of Controls we obtain a p-value of 0.550 and an effect size of 0,6.

Table 6 - Comparison of gender and age between AD patients and Controls of the study population.

	AD patients	Controls	p value	Effect Size (Cramer's V)
Total sample (n)	32	31	-	-
Men [n (%)]	10 (69%)	10 (68%)	0.932	0.011
Women [n (%)]	22 (31%)	21 (32%)		
Age (Mean ± SD)	76.0 ± 9.0	77.4 ± 9.2	0.550	0.6

4.2 Amplification of *BIN1* polymorphic region rs744373 by PCR

After checking the homogeneity between the two study groups (AD patients and Controls), we proceeded to the amplification of the *BIN1* polymorphic region rs744374 by PCR. There was no need to do prior extraction and quantification of genomic DNA since it was done directly by using Phusion Blood Direct PCR Master Mix (section 3.3.1).

After amplification the PCR products were precipitated and results were visualized in 1.5% agarose gels. The expected fragment of 767 bp was obtained in all cases, being a prerequisite to send the samples for subsequent sequencing. An example of a gel obtained by agarose electrophoresis is this study can be observed in Figure 8.

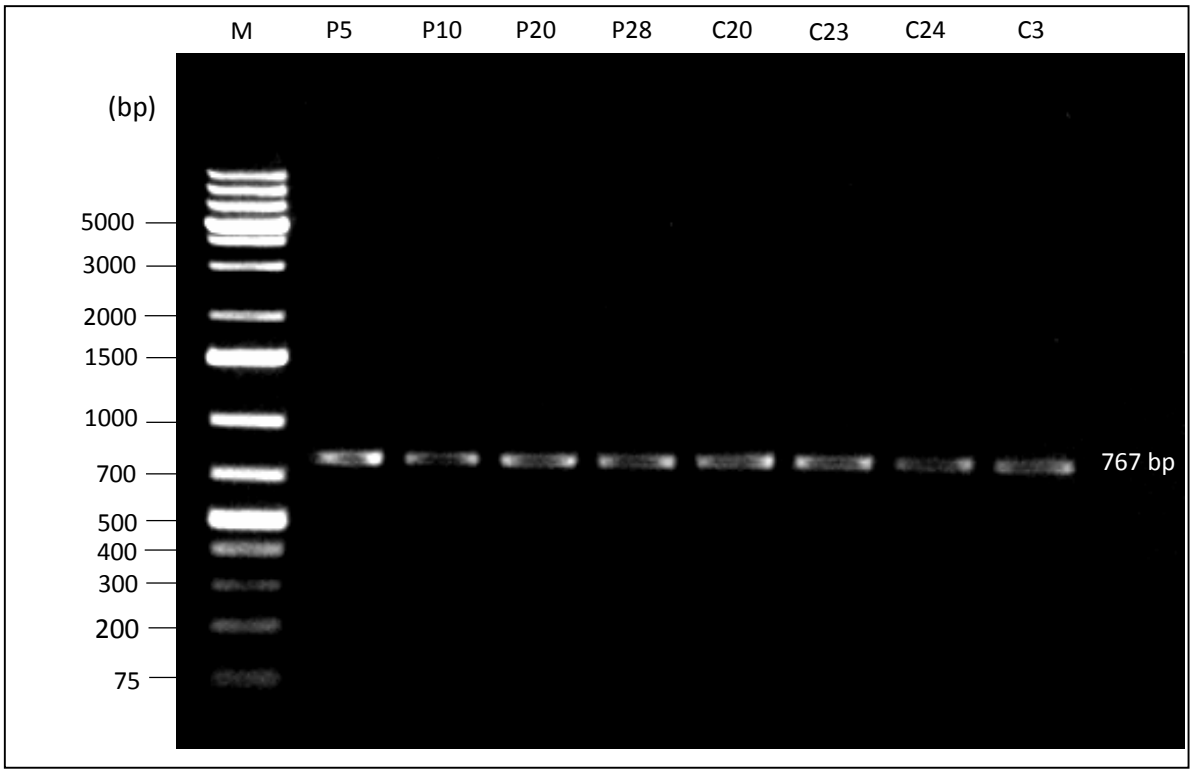

Figure 8 – *BIN1* polymorphism region amplified by PCR technique. The product band has 767 bp and was visualized in 1.5% agarose gel. Samples 337, 412, 484 and 534 belong to AD patients while samples 315, 362, 365 and 73 belong to Controls.

4.3 Correlating *BIN1* polymorphism rs744373 with AD

4.3.1 *BIN1* (rs744373) sequencing in the study group

After completing the precipitation of the PCR products, samples were submitted to Sanger Sequencing (section 3.3.4) and were subsequently analysed. Accordingly, individual genotypes based on the sequencing patterns were obtained, and examples shown in Figure 9.

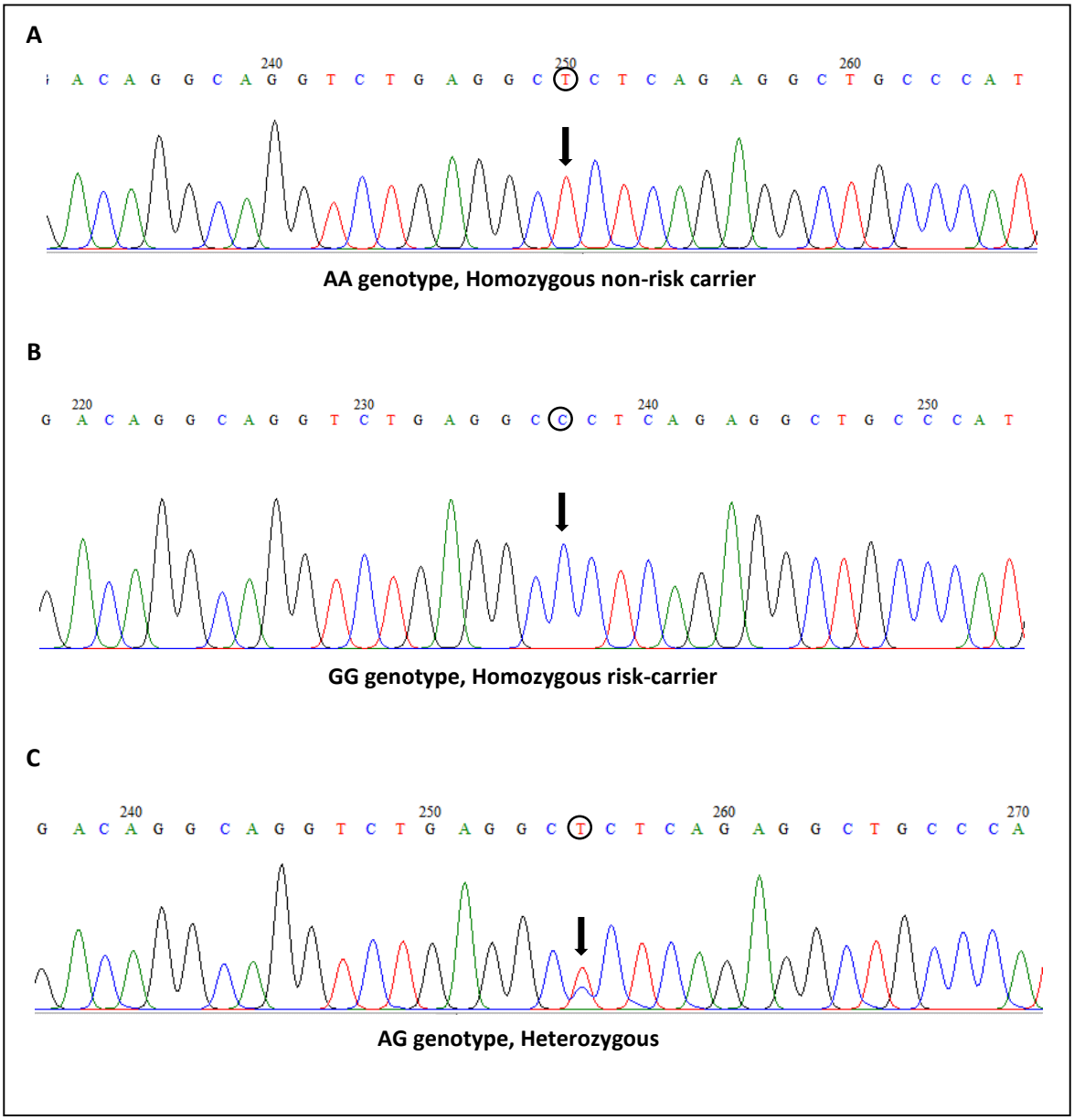

Figure 9 – Examples of Sanger-sequencing results of the *BIN1* gene rs744373 polymorphism, of the study population. A - AA genotype, Homozygous non-risk carrier. **B -** GG genotype, Homozygous risk carrier. **C –** AG genotype, Heterozygous.

Note that, since the primer used for Sanger sequencing (introduced in the 96-well plate sent for sequencing) was the BIN1-Rv and not the primer BIN1-Fw, the sequencing results here presented are shown having a C nucleotide instead of a G, and a T nucleotide instead of an A. Despite this detail, during this work the genotype of the different individuals will always be referred to as AA, AG or GG.

4.3.2 Genotype of the study population

After being collected and properly analysed, the sequencing results were organized in a table, so that it was possible to perform their subsequent genotypic and allelic analysis. Results are shown in Table 7.

Table 7 - Characterization of the study population according to gender, age and genotype.

AD PATIENTS				CONTROLS			
SAMPLE	GENDER	AGE	GENOTYPE	SAMPLE	GENDER	AGE	GENOTYPE
P1	F	56	AG	C1	F	74	AA
P2	F	74	AG	C2	F	81	AG
P3	F	83	AG	C3	F	62	AG
P4	M	74	AA	C4	F	75	AG
P5	F	70	AA	C5	M	60	AG
P6	M	74	AG	C6	M	74	AA
P7	F	78	AA	C7	F	83	AA
P8	F	77	AA	C8	M	85	AA
P9	M	88	AG	C9	F	71	AA
P10	F	73	AG	C10	M	77	AG
P11	F	85	AA	C11	F	75	AA
P12	F	78	AG	C12	F	78	AG
P13	M	81	AA	C13	F	73	AG
P14	F	78	AA	C14	M	75	AG
P15	F	95	AA	C15	M	74	AA
P16	F	75	AA	C16	M	81	AA
P17	M	78	AA	C17	F	79	AG
P18	F	82	AA	C18	M	58	AG
P19	F	84	AA	C19	M	77	AG
P20	F	91	AA	C20	F	81	AA
P21	F	86	AG	C21	F	84	AG
P22	M	87	AA	C22	F	76	AA
P23	F	88	AG	C23	M	88	AG
P24	F	76	AA	C24	F	56	AA
P25	F	80	GG	C25	F	77	AA
P26	F	80	AG	C26	F	71	AA
P27	M	60	AG	C27	F	80	AA
P28	M	58	AA	C28	F	81	AG
P29	F	71	AA	C29	F	93	AA
P30	F	62	AA	C30	F	87	AG
P31	M	79	AA	C31	F	79	AG
P32	M	75	AA				

4.3.3 Determination of the *BIN1* polymorphism rs744373 genotypic frequencies in the study population

Analysing the results present in Table 7, one can conclude that the AD patients group has 20 individuals with AA genotype (Homozygous non-risk carrier), 1 individual with GG genotype (Homozygous risk-carrier) and 11 individuals with AG genotype (Heterozygous, with 1 risk allele). On the other hand, the group of Controls shows 15 individuals with AA genotype and 16 with AG genotype, having no homozygous risk-carriers (GG genotype) (Table 8).

Table 8 - Number of individuals with each genotype and genotypic frequencies within the different groups.

GENOTYPE	GROUP					
	AD patients		Controls		AD patients + Controls	
	Nº Individuals	Genotypic Frequency	Nº Individuals	Genotypic Frequency	Nº Individuals	Genotypic Frequency
AA	20	62.5%	15	48.4%	35	55.6%
GG	1	3.1%	0	0.0%	1	1.6%
AG	11	34.4%	16	51.6%	27	42.9%
TOTAL	32	100%	31	100%	63	100%

According to these results, we proccedeed to determine the genotypic frequencies of each genotype within the different groups. Comparing the two there are small differences among them (Table 8 and Figure 10).

Starting with the AD patients group, the most frequent genotype was AA, having a genotypic frequence of 62,5%, followed by AG genotype (34,4%) and lastly GG genotype (3,1%). On the other hand, in the Controls group the genotype with higher genotypic frequence was AG (51,6%) followed by AA genotype (48,4%). In this group there were no individuals with the GG genotype.

Considering all individuals (AD patients + Controls), 35 out of 63 present an AA genotype (corresponding to a genotypic frequence of 55,6%), 1 individual has a GG genotype (1,6%), and 27 out of 63 individuals have AG genotype (42,9%). Thus, the order of the genotypic frequencies were: AA > AG > GG.

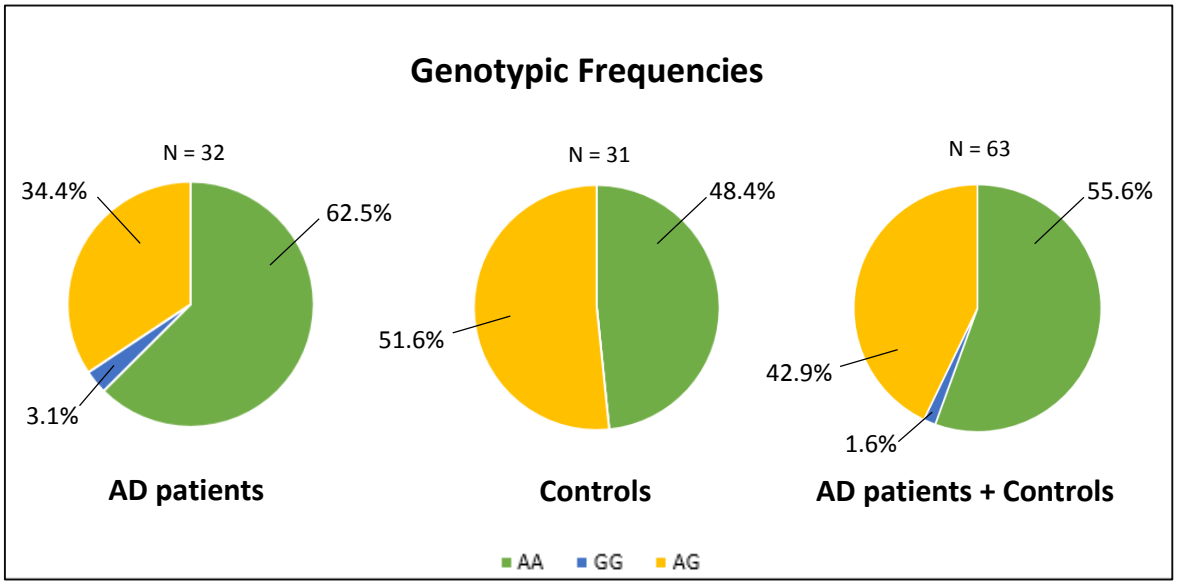

Figure 10 – Genotypic Frequencies in the different groups.

4.3.4 Determination of the *BIN1* polymorphism rs744373 allelic frequencies in the study population

Relatively to the allelic frequencies present in the different groups, in the AD patients group, allele A was the most frequent (51 alleles out of 64), while allele G represented only 13 alleles out of 64. Also, in the Controls group the most frequent allele was A (46 allleles out of 62) and allele G was the less frequent (16 alleles out of 62).

Therefore, when considering all individuals (AD patients + Controls), there is a big difference between the number of allele A and G present among the individuals, counting 97 alleles and 29 alleles, respectively (Table 9).

Table 9 - Number of each allele and allelic frequencies within the different groups.

ALLELE	GROUP					
	AD patients		Controls		AD patients + Controls	
	Nº Alleles	Allelic Frequency	Nº Alleles	Allelic Frequency	Nº Alleles	Allelic Frequency
A	51	79.7%	46	74.2%	97	77%
G	13	20.3%	16	25.8%	29	23%
TOTAL	64	100%	62	100%	126	100%

After calculating allelic frequencies within the different groups, we can observe a big difference between the two alleles. Allele A has a higher frequency in both AD patients group and

Controls group, representing 79,7% and 74,2%, respectively. On the other hand, G allele has only an allelic frequency of 20,3% in the AD patients group and 25,8% in the Controls group.

Looking for a global context, and considering all individuals, the allelic frequency of A allele is 77% against the 23% of the G allele (Figure 11).

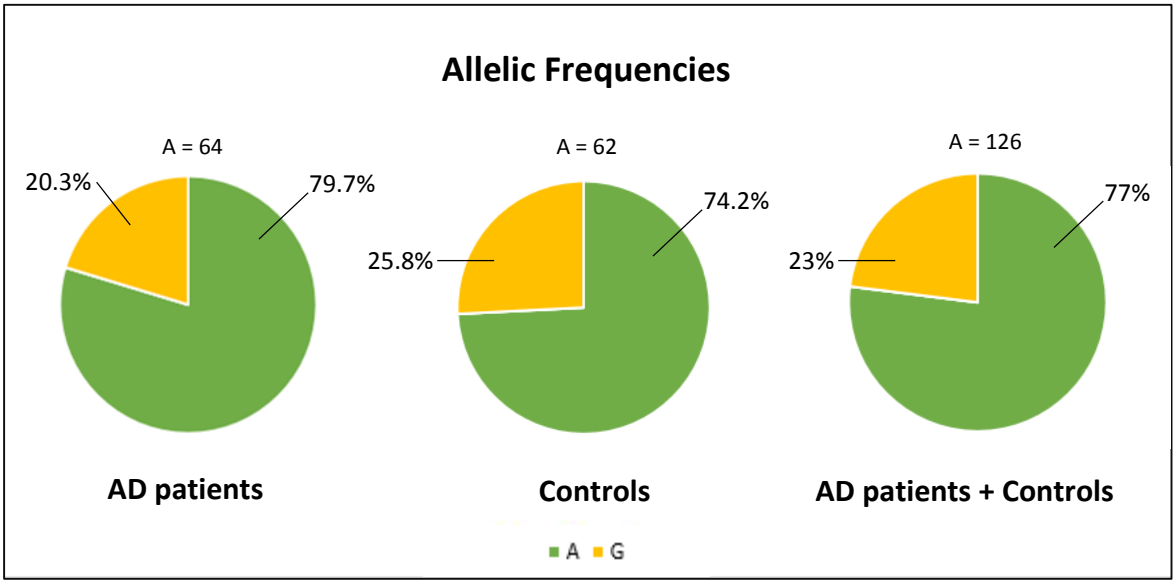

Figure 11 – Allelic Frequencies within different groups.

4.3.5 Evaluation of the statistical significance of rs744373 in the study population

One of the aims of this work was to evaluate the statistical significance of *BIN1* polymorphism rs744373 in the study population. Therefore, according to the genotypic frequencies present in the AD patients group and in the Controls group, the p-value and effect size were calculated in order to assess possible differences between the two groups. Thus it is possible to evaluate whether the genotype that confers higher risk for developing Alzheimer's Disease (GG genotype) is present in a substantially greater quantity in AD patients than in Controls and, on the other hand, if the genotype that confers a lower risk of developing the disease (AA genotype) is higher in controls when compared to AD patients. If this scenario were to happen, it could mean that the analysed polymorphism constitutes a risk factor to developing AD, in the study population.

Comparing the AD patients group and the Controls group the p-value was 0.260 and the effect size was only 0,20 (Table 10), showing that the difference is not statistically significant between the two analysed groups.

Table 10 - Comparison between the genotype of AD patients and Controls of the study population.

	AD patients			Controls			p value	Effect Size (Cramer's V)
Genotype (Nº Individuals)	AA	GG	AG	AA	GG	AG	0.260	0.20
	20	1	11	15	0	16		

In order to assess if there were significant differences among the two groups and if the presence of the risk allele (also denominated Minor allele) of the *BIN1* polymorphism rs744373 (allele G) was significantly higher in the AD patients group rather than the Controls group, we performed an association test between the two groups (Table 11). With this test we intended to evaluate if the rs744373 could be considered as a risk locus to develop LOAD, in this particular population.

Table 11 - Association of rs744373 with the AD risk in the study population.

	Number of individuals		MAF		Association test	
	AD patients	Controls	AD patients	Controls	OR(95% CI)	p-value
rs744373	32	31	0.203	0.258	0.733 (0.318–1.686)	0.464

Key: MAF, minor allele frequency; OR, odds ratio; CI, confidence interval.

Comparing the AD patients group and the Controls group we didn't find strong evidence of association for rs744373 with the AD risk (odds ratio [OR] = 0.733 , p-value = 0.464).

4.3.6 Association of rs744373 with APOE-Ɛ4 carriers and CDR

One of the goals of this work was to assess if there was an association between APOE-Ɛ4 carriers and *BIN1* polymorphism rs744373 as well as an association between rs744373 and CDR (Table 12).

Overall, we can observe that, in the first situation, most of the study subjects are non-Ɛ4 carriers (84.1%). Of those, 31 have an AA genotype, 21 have an AG genotype and only one has a GG genotype. On the other hand, Ɛ4 carriers count for 10 out of 63 of the total study population. Of those, 4 have an AA genotype and 6 have an AG genotype, but none with respect to the main risk genotype (GG).

No statistical significance was detected between rs744373 and APOE-Ɛ4 carriers (p-value = 0.467).

Table 12 - Association of rs744373 with APOE-Ɛ4 (carriers and non-carriers) and CDR in the study population.

SNP	Genotype	APOE-Ɛ4		Association test	CDR		Association test
		Non-Ɛ4 carriers	Ɛ4 carriers	p-value	CDR=0 Normal	CDR≥1 Dementia	p-value
rs744373	**AA** [n/n total AA genotype (%)]	31/35 (88.6%)	4/35 (11.4%)	0.467	15/35 (42.9%)	20/35 (57.1%)	0.269
	AG [n/n total AG genotype (%)]	21/27 (77.8%)	6/27 (22.2%)		16/27 (59.3%)	11/27 (40.7%)	
	GG [n/n total GG genotype (%)]	1/1 (100%)	0/1 (0.0%)		0/1 (0.0%)	1/1 (100%)	
	Total/ Total study population	**53/63 (84.1%)**	**10/63 (15.9%)**	-	**31/63 (49.2%)**	**32/63 (50.8%)**	-

Relative to the correlation between rs744373 and CDR, the number of normal individuals (CDR=0) and individuals with dementia (CDR≥1) are very similar (31 against 32, respectively). In the group of CDR=0, 15 have an AA genotype and 16 have an AG genotype. In this group there are none homozygous risk-carrier (GG genotype). On the other hand, in individuals with dementia (CDR≥1), 20 have an AA genotype, 11 have an AG genotype, and only one has a GG genotype.

No statistical significance was detected between rs744373 and dementia (CDR≥1) (p-value = 0.269).

4.3.7 Association of rs744373 with Diabetes Mellitus

Since AD is such a multifactorial disease, we wanted to evaluate if the polymorphism studied in this work was related to some comorbidities such as Diabetes Mellitus, hypertension and gastrointestinal disease. Of these only the first showed an association with rs744373 (Table 13) and so, the results for other comorbidities are not presented in this work.

Table 13 - Association of rs744373 with Diabetes Mellitus in the study population.

SNP	Genotype	Diabetes Mellitus		Association test	
		No	Yes	p-value	OR (95% CI)
rs744373	**AA** [n/n total AA genotype (%)]	33/35 (94.3%)	2/35 (5.7%)	0.035	6.60 (1.27-34.23)
	AG [n/n total AG genotype (%)]	19/27 (70.4%)	8/27 (29.6%)		
	GG [n/n total GG genotype (%)]	1/1 (100%)	0/1 (0.0%)		
	Total/ Total study population	**53/63 (84.1%)**	**10/63 (15.9%)**	-	-

In the study population, Diabetes Mellitus was present in 10 individuals and absent in 53. Most of the subjects which had Diabetes present AG genotype and only 2 had an AA genotype. On the other hand, in individuals without Diabetes, AA genotype was the most frequent, followed by AG genotype. The main risk genotype (GG) was only present in 1 individual, which had no Diabetes.

A significant association between rs744373 and dementia Diabetes Mellitus (odds ratio [OR] = 6.60, p-value = 0.035) was detected.

5. DISCUSSION AND CONCLUSION

In the last few years, with the world's population rapidly aging, and with the extended life spans, Dementia is becoming increasingly common, representing one of the major factors driving of costs in the health care and social systems, thus being a major health and socio-economic problem worldwide. Although there are several types of dementia, AD is the most common and for this reason, it becomes increasingly imperative to understand the genetic and molecular characteristics associated with the disease, so that it is possible to establish early diagnostic methods and to develop therapeutic strategies that allow to delay its progression. In addition, till present the definite diagnosis of AD is only possible by neuropathological examination at autopsy or biopsy and so there is a lot of pressure to find new ways to diagnose AD based on biomarkers and genetic tests. Over the years, several studies have focused on finding answers to these problems, however a lot questions still remain [169,170]. Also, since AD is such a multifactorial disease, understanding the interactions among genetic, epigenetic and environmental factors and pathways that might be linked can provide useful information concerning ways of controlling this dementia [5,15].

Regarding the genetics of AD, recently *BIN1* has been recognised as the most significant LOAD-associated risk loci after APOE. Several functions have been described for *BIN1*, such as a role in the process of clathrin-mediated endocytosis as well as in regulating the actin cytoskeleton [107] leading to many possible associations of this protein to rare and common diseases affecting different tissues and physiological functions [110]. Relative to AD, several studies evaluated the potential role of this BAR protein in APP metabolism and Aβ production but failed to find any association between the levels of BIN1 and APP processing [139] or Aβ42 neurotoxicity [138]. Further, BIN1 have been associated with TAU pathology, since it might modulate microtubule stability or else TAU phosphorylation/aggregation [138], influencing the early stages of AD. However, these findings are not completely accepted by the scientific community and so, further studies should focus on answering some of these questions.

Moreover, several GWAS have been performed by several groups and although many SNPs of *BIN1* have been linked to LOAD, little is known about BIN1 protein expression and its contribution to AD pathogenesis. Among those, rs744373 SNP (risk allele G) was one of *BIN1* polymorphisms that was especially relevant for AD [109,129]. Nevertheless, it was still not possible to draw conclusions about the significance of this polymorphism in different types of populations and the number of individuals that has been analysed is small to achieve this level of significance. Thus it is imperative

that more studies are performed in order to help clarify the importance of *BIN1* to LOAD in different groups of individuals with different ethnicities.

In this perspective and since, to our knowledge, no studies have been done in order to assess the relevance of *BIN1* polymorphism rs744373 as a risk locus for AD, in a Portuguese population, the work here presented aimed to investigate the *BIN1* profile, related to rs744373, in a pilot study group of 63 individuals (32 potential AD patients and 31 controls) from a cross-sectional population-based study on a Portuguese population, in the Aveiro district.

5.1 Assessment of the balance between the two study groups

Since AD is a multifactorial disease which usually affects people over 65 years old and predominantly female, in this type of study it is important to evaluate if the groups to be studied are balanced, particularly regarding both gender and age, so the results are not influenced by significant differences between the two groups with respect to these two factors. The individuals of the study were divided into two groups: AD patients and Controls. The first group had 22 females and 10 males, while the second has 21 females and 10 males (p value = 0.932 ; effect size = 0.011). The mean age of AD patients was 76 years and in Controls it was around 74 years (p value = 0.550 ; effect size = 0.6). According to these results, we can conclude that there are no significant differences between the two study groups, therefore it is a balanced population. Note that, since *BIN1* is believed to be associated with LOAD [129] and not to EOAD, we were careful to try to select individuals aged over 65 years old.

5.2 Determination of *BIN1* polymorphism rs744373 genotypic frequencies in the study population

In order to amplify *BIN1* polymorphic region rs744373, PCR reactions were carried out. After this was performed a precipitation of the PCR products which were later subjected to Sanger sequencing. Genotypic and allelic frequencies of rs744373 SNP were determined for AD patients and Controls, as well as the genotypic and allelic frequencies for all study subjects. Considering the AD patients group, the genotype frequencies were as follows: AA > AG > GG, with 62,5%, 34,4% and 3,1%, respectively. The results of the Control group were slightly different, with the highest

genotypic frequency corresponding to AG (51,6%), followed by AA (48,4%). The GG genotype was absent in this group. Overall, considering all individuals of the study, the most frequent genotype was AA and this was found to be 35 times higher than the less frequent genotype, which was GG. This results lead us to believe that *BIN1* polymorphism is not a risk factor for this specific Portuguese population, since the non-risk genotype (AA genotype) is present in most of the individuals in the study, while the genotype which confers the greater risk (GG genotype) is only present in one individual in both groups (found in 1 AD patient).

5.3 Determination of *BIN1* polymorphism rs744373 allelic frequencies in the study population

Regarding the rs744373 SNP allelic frequencies, we can observe a major difference between the A allele and the G allele, being that the first has an overall allelic frequency of 77%, while G allele has an allelic frequency of only 23%. Thus, in this study population, the A allele is 3 times more frequent than allele G. Looking at the two study groups separately, one can observe that the results are very similar between the groups (79,7% AA and 20,3% GG in AD patients, against 74,2% AA and 25,8% GG in Controls). Once again, these results emphasize the low presence of the risk allele G in this study population, besides showing that differences between groups are not very evident, indicating that the *BIN1* polymorphism rs744373 is not relevant for the onset and development of LOAD, for this particular set of individuals.

5.4 Evaluation of the statistical significance of rs744373 in the study population

Subsequently the statistical significance of rs744373 in the study population was evaluated. First it was found that there are no significant differences among genotypes between the two study groups (p-value = 0.260, Cramer's V = 0,20). In addition, the results do not seem too promising since we did not find strong evidence of association for rs744373 with the AD risk (odds ratio [OR] = 0.733 , p-value = 0.464). When consulting the literature (Table 14), it is evident that these results are consistent with studies previously performed in different populations such as the Finish population[132], the African American[135] and the Han Chinese population [136], which suggest that *BIN1* is not necessarily associated with AD development. Despite this, although our study failed to do so,

other studies were able to replicate the results of Seshadri *et al.*, which was the first group providing compelling evidence that the polymorphism rs744373 is associated to LOAD (p value = $1.59x10^{-11}$), identifying *BIN1* as a risk factor for AD. Moreover, studies performed in Spanish populations and in an Italian population found strong evidence of the association between *BIN1* (rs744374) and an augmented risk of developing AD. In fact, a study performed by J.-C. Lambert *et al.* showed that, regardless of having two significant associations (Spanish and Italian populations) and a not significant result (Finish population), overall, when performing a meta-analysis, there was a high significance of *BIN1* polymorphism rs744373 to AD (p-value = $2.9x10^{-7}$). Once again, these results emphasize the need to carry out more of these studies, since only one study in each population proves to be insufficient to draw conclusions about the significance of a gene with respect to the onset or development of AD.

Table 14: **Association of *BIN1* polymorphism rs744373 with AD in different study populations.**

SNP	Minor Allele	Population	No. Subjects		MAF	Association Test		Ref.
			Cases	Controls		p-value	OR(95% CI)	
rs744373	G	Causasian	8371	26969	0.29	$1.59x10^{-11}$	1.15 (1.11-1.20)	129
		Spanish	1140	1209	0.29	0.020	1.17 (1.03-1.33	129
		Spanish	726	829	0.29	$1.4x10^{-7}$	1.43(1.22-1.68)	132
		Italian	1460	1265	0.29	0.002	1.22 (1.07-1.38)	132
		Finish	563	529	0.24	0.260	1.12 (0.92-1.37)	132
		African American	513	496	0.48	0.999	1.00 (0.84-1.20)	135
		Han Chinese	612	612	0.35	0.217	1.11 (0.940-1.312)	136
		Portuguese	**32**	**31**	**0.23**	**0.464**	**0.733 (0.318-1.686)**	**This study**

Key: MAF, minor allele frequency; OR, odds ratio; CI, confidence interval; Ref, Bibliographic reference.

In this work we failed to confirm the association of *BIN1* polymorphism rs744373 with AD risk. However, the lack of replication of the results obtained by Seshadri *et al.* might be due to the small sample size, when compared to other consortium-based GWAS. Also, with such a small population this study turns out to have no power to detect rare variants with weak effects. Furthermore, the absence of replication of the original findings could be due to type I error (ie, false-positive results in the original study). Despite this conclusion, it would be extremely interesting and relevant to find an association between *BIN1* polymorphism rs744373 and AD with such a small population size since, if we had found an association between the two, we could hypothesize that this SNP could

be used for diagnosis purposes, since a simple genetic analysis of this locus could determine the propensity of an individual to develop AD.

Despite the small size of our sample, other studies performed in larger samples also reached the conclusion that rs744373 is not relevant to AD, which may confirm that SNPs which confer risk to onset and development of AD are specific to each population. With our results, rs744373 would not be specific for the Portuguese population, although other SNPs could contribute to AD risk. For this reason, it is imperative to reproduce this study in a larger population, as well as investigate the possible association of other polymorphisms of *BIN1* in the Portuguese population, in order to better understand the genetic behind AD.

Still, there may be another reason to justify the lack of significance of this polymorphism in our study population. According to B. Jiao *et al.* the SNP rs744373 (allele A) might have a protective effect against AD in a Chinese population [171]. Nevertheless, this is still poorly explored and so, according to the lack of significance in our results for the Portuguese population, it might be possible that in these specific individuals rs744373 exerts a protective effect, however additional studies in larger Portuguese populations are required to determine if this can be a likely explanation.

Importantly, despite the results and literature herein presented (Table 14), it is important to have in mind that these results do not mean that *BIN1* has no association with AD. In fact, several other SNPs in this candidate gene, identified from previous GWAS, have been linked to the disease. An example can be found in the study performed by Logue *et al.*, which revealed that, despite rs744373 not having an association with AD in African Americans, in the same study population rs11685593, rs11691237 and rs7585314 were significant with a p-value of 0.0098 to the first two SNPs and a p-value of 0.003 to the latter [135]. This highlights that the most significant SNPs may vary within different populations and that more than one SNP may be associated to the development of AD, emphasizing the importance of continuing to perform further studies of this nature in more types of populations, and even in populations which have already been analyzed, by increasing the number of SNPs to be analyzed in each population. Also, the greatness of the effect of a risk allele may differ among populations since gene-gene or gene-environment interactions may be exerting an effect in the study individuals and so future studies should continue to assess possible interactions between SNPs.

5.5 Association of rs744373 with APOE-Ɛ4 carriers and CDR

Another goal of the current work was to identify whether rs744373 was associated with APOE-Ɛ4 allele or CDR≥1, in our study population. We first analyzed the association between the polymorphism and APOE-Ɛ4 carriers. However, no association was detected (p-value = 0.467). Since *BIN1* as a role in clathrin-mediated endocytosis and in intracellular endosome trafficking, it would be expected to be associated with APOE. Despite this, our results show otherwise. Nevertheless, just because rs744373 does not appear to be related to the risk allele of that apolipoprotein, it does not mean that *BIN1* has no association, since other SNPs may be associated to that protein. Therefore, more studies should be done in order to evaluate if BIN1 interacts with APOE, resulting in a higher probability of developing AD.

Relatively to CDR, we wanted to analyse if, in our study population, subjects with dementia had a greater chance of having the presence of the risk allele of rs744373 (G allele). If we would have found that association, it would be an asset for diagnosis purposes, since the detection of the risk allele in an individual could mean that this person was more likely to develop dementia in the future. However, the current study did not detect an association between CDR≥1 individuals and rs744373 risk allele (p-value = 0.269). Nevertheless, as described above, the lack of association found in our results could be due to sample size, which was not large enough to detect extremely weak interactions.

5.6 Association of rs744373 with Diabetes Mellitus

The last goal of this work was to study the association of the gene variant rs744373 with Diabetes Mellitus (DM). AD is a multifactorial disease, affected by genetic, epigenetic and environmental factors. As previously stated, some of the non-genetic factors that might contribute to AD include infections, hormones or even Diabetes [16].

In addition, studies suggest that the increased familial risk in African Americans is probably a result of higher rates of risk factors, including poor education, DM, and smoking [135]. Our results are consistent with this literature, since rs744373 risk allele seems to be associated with the presence of DM (odds ratio [OR] = 6.60, p-value = 0.035). Thus, in this study population, individuals with diabetes are 6.60 times more likely to have the risk allele of *BIN1* polymorphism (G allele) and, therefore more likely to develop AD. A follow-up study, in a larger population should be addressed,

in order to investigate if these results can be generalized to the Portuguese population, or if they are limited to our study population.

5.7 Final conclusions

Although this study represents an important step in elucidating the genetic basis of AD in a Portuguese population, and offers some understanding for population-specific genetic risk factors, it has some restrictions. First, the small sample size of this study may be underpowered to detect the small effects of this *BIN1* polymorphism (rs744373) on AD risk. Likewise, since it was beyond the scope of this study, we did not study gene-gene or gene-environment interactions, which would be an asset.

Most association studies have focused on a single population in order to decrease genetic background noise and reduce the likelihood of false-positive findings. For this reason, validation in additional populations is mandatory to conclude the generalizability of the contribution of *BIN1* gene to AD risk and the possibility of population-specific causative variants.

CONCLUDING REMARKS:

- Of the three possible genotypes for *BIN1* polymorphism rs744373, in the AD patients group AA genotype had highest frequency, while in Controls group the most frequent genotype was AG.
- In both study groups GG genotype was the one with the lowest frequency.
- In our study population A allele is 3 times more frequent than allele G (risk allele), indicating that rs744373 doesn't confer risk to AD.
- We did not find strong evidence of association for rs744373 with the AD risk (odds ratio [OR] = 0.733 , p-value = 0.464), in agreement with other studies. This supports the fact that it is important to carry out a follow up study to clarify these results.
- In our study population the most significant SNP may be a SNP other than rs744373, associated to the development of AD. To answer this, further studies are required.
- We did not detect an association between rs744373 and APOE-Ɛ4 carriers or CDR≥1 individuals.
- An association between rs744373 risk allele and the presence of DM was evident. Nevertheless, due to our small sample size a follow-up study in a larger sample should be performed to evaluate if these results can be generalized to the Portuguese population.

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7. ANNEXES

This section presents the reagents as well as the equipment necessary for the several techniques used during this work.

7.1 Polymerase Chain Reaction (PCR)

EQUIPMENT

- Thermocycler Eppendorf® Mastercycler (Sigma-Aldrich, USA)
- Spectrafuge Mini Centrifuge (Labnet International, USA)

REAGENTS

- 2XPhusion Blood Direct PCR Master Mix (ThermoFisher Scientific, USA)
- Primers (Eurogentec, Belgium)
  - *BIN1*-Fw: 5' AAG ACG GAG AGA GGA GGC AT 3'
  - *BIN1*-Rv: 5' CCA TCT TCT TCT GCT CTC CCA G 3'
- Ultrapure DNase/RNase-free distilled H_2O (ThermoFisher Scientific, USA)

7.2 Precipitation of DNA fragments

EQUIPMENT

- 5417R Mini centrifuge (Eppendorf AG, Hamburg)

REAGENTS

- Sodium Acetate (pH 3M, pH 5.2)

  To 80mL of deionized H_2O add:

  - 24,6g Sodium Acetate, minimum 99,0% (Sigma-Aldrich, USA)

  Mix until the solute has dissolved and adjust pH to 5.2 with Glacial Acetic Acid.
  Adjust the volume to 100mL with deionized H_2O. Store at room temperature.

- Ethanol 100% (Aga, Portugal)
- Ethanol 70% (Aga, Portugal)
- Ultrapure DNase/RNase-free distilled H_2O (ThermoFisher Scientific, USA)

7.3 Agarose Gel Electrophoresis

EQUIPMENT

- DNA electrophoresis system (Bio-Rad Laboratories, Inc., USA)
- PowerPAC™ Basic 300V (Bio-Rad Laboratories, Inc., USA)
- Molecular Imager® Gel Doc™ XR+ (Bio-Rad Laboratories, Inc., USA)

REAGENTS

- Agarose (Nzytech, Portugal)
- Bromophenol Blue

  To 7mL of deionized H_2O add:

  - 0,025g bromophenol blue (0,25%)
  - 3mL glycerol (30%)

  Mix and store at 4°C.

- Greensafe (Nzytech, Portugal)
- 0,5M Ethylenediamine tetra-acetic acid (EDTA) (pH 8.0)

  To 80mL of deionized H_2O add:

  - 14,612g EDTA

  Mix until the solute has dissolved and adjust pH to 8.0 with NaOH. Adjust the volume to 100mL with deionized H_2O. Store at room temperature.

- 50X TAE (Tris-Acetate-EDTA Buffer)

  To 600mL of deionized H_2O add:

  - 242g Tris Base
  - 57,1mL Glacial Acetic Acid
  - 100mL 0,5M EDTA (pH 8.0)

  Mix until the solutes have dissolved and adjust the volume to 1L with deionized H_2O. Store at room temperature.

- 1kb Plus DNA Ladder (ThermoFisher Scientific, USA)